STUDY OF HEPATIC CYTOCHROME P450 SYSTEM IN RICHARDSON GROUND SQUIRRELS

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ABSTRACT

Richardson ground squirrels (gophers) are pests on the prairies that cause considerable agricultural and ecological damage. Traditional control methods such as the rodenticides strychnine, zinc phosphide, and anticoagulants, have proven ineffective in reducing gopher densities. In additional, current gopher control methods have the significant potential to cause primary and secondary toxicity to non-target animals. Thus, alternative methods for toxicological control of gophers are needed to mitigate these concerns. Present studies focused on the cytochrome P450 (CYP450) enzyme system responsible for xenobiotic detoxification in gophers. *In vitro* hepatic microsomal systems and HPLC analysis were used to elucidate general metabolic characteristics of major gopher xenobiotic metabolizing pathways. We found that the content and activity of individual components of the CYP450 system including CYP450, cytochrome b5, and NADPH-cytochrome P450 reductase in liver microsomal preparations were higher in gophers exposed to toxins used to control their population than in naïve (unexposed) gophers. When *in vitro* CYP450 mediated activities for five substrates [coumarin and aniline aromatic hydroxylation, 7-methoxycouamrin O-demethylation, and N-methylaniline, and N,N-dimethylaniline N-demethylation] were measured, naïve gophers were identified to have higher specific activity but similar whole body activity compared to the exposed gophers. Furthermore, there was a clearly identifiable sub-population of "poor metabolizers" showing considerably lower CYP450 activity within the gopher samples studied. Clotrimazole was found to be a potent inhibitor of several substrates of CYP450 enzyme-mediated reactions, which included aniline aromatic hydroxylation, N-methylaniline and N,N-dimethylaniline N-demethylation, and 7-methoxycoumarin O-demethylation. The cytotoxicity of above compounds was tested using freshly isolated gopher hepatocytes. The results showed that each compound caused considerable cytotoxicity to gopher hepatocytes. Addition of clotrimazole to the freshly isolated hepatocyte suspension increased the cytotoxicity of all tested compounds.

In conclusion, gophers may develop resistance to current chemical control methods through the enhancement of CYP450 system content, which can compensate the loss of enzyme activity. Furthermore, clotrimazole is a potent cytochrome P450 inhibitor, which increases the cytotoxicity caused by given compounds in gopher livers. The concept of using CYP450 enzyme inhibitor in combination with another chemical whose elimination depends on CYP450 metabolism to improve current gopher control method has practical importance.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

^0C	degree of celsius
7-HC	7-OH-coumarin
7-MC	7-methoxycoumarin
ANOVA	analysis of variance
BW	body weight
Cl_{int}	intrinsic clearance
cm	centimeter
CTZ	clotrimazole
CV	coefficient of variation
CYP450	cytochrome P450
DMA	N,N-dimethylaniline
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetracetic acide
E_m	emission wavelength
E_x	excitation wavelength
g	gram
h	hour
HPLC	high performance liquid chromatography
IC	inhibitor concentration
IMD	Iscove's Modified Dulbecco's medium

KCl	potassium chloride
kg	kilogram
K_i	enzyme inhibition constant
K_m	Michaelis-Menten constant
L	liter
LD_{50}	lethal dose for 50% of the animal test population
LSD	least significant difference
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MP	mobile phase
MS	microsomal protein
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
NMA	N-methylaniline
nmol	nanomolar
P - value	probability of significance
pH	potential of hydrogen

PM	poor metabolizer
R^2	coefficient of determination
RT	retention time
SE	standard error
SM	strong metabolizer
V	velocity
V_{max}	maximal velocity

1.0 INTRODUCTION

Richardson's ground squirrels (*Spermophilus richardsonii*), commonly known as gophers, are ubiquitous throughout the mixed-grasses and fescue prairies in North America, including some portions of Canada like Saskatchewan, Alberta, and Manitoba.

Gophers are considered an integral component of the prairie ecosystem. They provide an important source of food for wildlife like eagles, owls, hawks, and red foxes. Therefore, some people think they are obligate animals that should be preserved for the benefit of the ecosystem. However, in most instances gophers are considered as a pest animal. Since people came to dwell on the prairie they have been trying to control the population of gophers due to their considerable agricultural and ecological damage and their potential hazard to livestock and humans. Historically, measures used to control gopher populations included trapping, fumigation, shooting, and chemical control using various poison baits such as strychnine, zinc phosphide, and anticoagulants (Matschke et al., 1983; Schmutz et al., 1989; Farm Facts). Because of its practicality, chemical control of gophers has been a method of choice for several decades.

A paucity of scientific information exists on gopher population changes. However, a recent survey from Saskatchewan (2001 Saskatchewan "Gopher" Survey) suggests gopher populations have increased over the last decade. This indicates that the traditional methods of chemical and non-chemical gopher populations control are ineffective. This apparent lack of success in controlling gopher populations using traditional chemical methods may be a result of the Richardson ground squirrels' systemic adaptation to toxins used for its control. The concern over resistance to chemical controls is further compounded by the increasing primary and secondary hazards associated with non-target species like predator or scavenger animals who consume baits or gopher carcasses.

The toxins used to control gopher populations are delivered by oral administration, which will be further biotransformed in gopher body. The biotransformation of toxins in the body is for the purpose of alleviating toxicity and to eliminate compounds from the body. Metabolism of toxins involves two distinct types of enzymatic reactions: 1) phase I reactions, which modify the native structure of a parent compound via oxidation, reduction, and hydrolysis reactions; and 2) phase II reactions that conjugate either modified or intact parent compound to ligands such as glutathione, glucuronic acid, amino acids, and sulphate. Phase I reactions play an important role in alleviating acute toxicity or (in some instances) can produce metabolites that may pose greater toxicity than the parent compound. Major phase I reactions are performed by enzyme systems embedded in the endoplasmic reticulum, commonly known as cytochromes P450. Cytochromes P450 comprise a super-family of enzymes involved in the metabolism of endogenous and exogenous compounds (Klassen, 2001, Cytochrome P450 homepage).

Selected aspects of hepatic cytochrome P450 detoxification system and its specific activity in gophers were examined in this project. Liver microsomal fractions were used as an *in vitro* model to evaluate the cytochrome P450 (CYP450) system in gophers, which is the major enzyme system responsible for detoxification of ingested xenobiotics. The measurements included: 1) CYP450 content, 2) Cytochrome b5 content, and 3) NADPH-Cytochrome P450 reductase activity. Several substrates including N,N-dimethylaniline, N-methylaniline, coumarin, aniline and 7-methoxycoumarin were used as potential probes for biotransformation reactions including N-demethylation, aromatic hydroxylation and O-demethylation. Furthermore, the potency of a general CYP450 inhibitor clotrimazole (CTZ) on the selected substrates metabolism was evaluated. Freshly isolated hepatocytes were used to test the toxicity of these selected substrates as model of a whole cell system.

1.1 Hypothesis

Metabolic functions responsible for detoxification of xenobiotics show substantial inter- and intra-species differences (Lewis et al., 1998). We hypothesize that there are some inter-species differences between gophers and non-target wildlife as well as intra-species difference between the male and female gophers in their capacity for xenobiotic metabolism. These features may be exploited to design better means to control gopher population.

The cytochrome P450 hepatic mixed function oxidase system plays a crucial role in determining the magnitude and duration of toxic effects of a wide variety of xenobiotics (Guengerich, 1997). A study of the biotransformation of selected substrates in gophers may be performed using probes representing the common biotransformation reactions performed by the CYP450 enzymes. Xenobiotic metabolism using an *in vitro* model may reveal differences in CYP450 levels, metabolic profiles, and response to inhibitors. Therefore, we hypothesize that *in vitro* experiments of xenobiotic metabolism can be extrapolated to make predictions about *in vivo* xenobiotic metabolism in gophers.

The data so obtained will reveal general trends if species specific characteristics exists. This knowledge may be further exploited to design a better alternative for toxicological control of gophers.

1.2 Objectives

General Objectives

The overall goal of this study was to define potential metabolic weaknesses of the enzymatic pathways responsible for xenobiotic detoxification in gophers. Such information may lead to design and development of new, more effective, species specific and ethically acceptable technologies to control populations of gophers.

Specific Objectives

1. To evaluate the main detoxification system (cytochrome P450 system) in gophers using liver microsome fractions from previously exposed and naïve gophers.
2. To characterize Cytochrome P450 activity representing several fundamental generic groups of biotransformation reactions with emphasis on aromatic hydroxylations, O-dealkylation, and N-dealkylation using selected compounds as probes.
3. To characterize enzyme kinetics of cytochrome P450 mediated biotransformation [Michaelis-Menten constant (K_m) and the maximal velocity (V_{max})] for selected substrates.
4. To investigate the effects of a general CYP450 inhibitor on the specific activities of CYP450 enzymes and determine IC_{50} (concentration causing 50% inhibition).
5. To evaluate the cytotoxicity of selected substrates using freshly isolated hepatocytes from gophers in the presence and absence of a general CYP450 inhibitor.

2.0 LITERATURE REVIEW

2.1 General Aspects of Pest Control

Since the beginning of agriculture, farmers have been faced with the burden of how to deal with and control pests. Pests can be defined as unwanted plants, animals or microorganisms. Currently, there are two different methods of pest control, chemical and non-chemical methods. Non-chemical methods stress cultural, mechanical and biological controls as well as the strict avoidance or limited use of chemical baits. These methods include trapping, shooting, and the use of predatory organisms e.g. bacteria, fungi, plants, or animals. Despite the availability of alternative methods of pest control, the use of chemicals plays an important role in integrated pest management. The principle reason for this is that other methods of pest control may not be available to all agriculturalists due to expense or lack of effectiveness. Pesticides, however, have allowed farmers to dramatically increase yields with less time and labor consumptions.

Effective pesticides exhibit three important characteristics: 1) high efficacy at low rates of applications, 2) selective toxicity to specific pest species, and 3) low toxicity to beneficial organisms and the environment. However, it should be noted that the uses of pesticides carry a variety of associated risks to humans and the environment (NRC, 2000). For instance, exposure to pesticide residues in drinking water (Harrison et al., 2000) and diets (Vongbuddhapitak et al., 2002) trigger strong public concerns about its potential toxicity to humans.

Rodents, belonging mainly to the families Sciuridae (Tree and ground squirrels), Cricetidae (voles, gerbils, and hamsters), and Muridae (rats and mice) are considered as pests in both commercial and agricultural situations (Muller, 2000). These animals are considered to be one of the most damaging pests to crops (Muller, 2000). Almost all food produced for consumption by both humans and domestic animals are liable to be

damaged by rodents during processing and storage, leading to significant financial losses. Furthermore, they constitute a high health risk to both livestock and humans, since rodents may transmit diseases to humans and domesticated animals either directly through bites or through exposure to infected feces and urine, or by indirect means via a vector (usually an arthropod such as a fly or mosquito) (Gage and Kosoy, 2005). Their potential to cause significant economic loss and pose a health risk have lead to numerous rodent control techniques including both chemical and non-chemical methods. The difficulty with these methods is that they pose potential primary and secondary toxicity to non-target animals including farm animals and humans. As a result, animal welfare of both target and non-target animals must be considered when devising chemical control methods.

2.2 Richardson's Ground Squirrels

Named after the naturalist John Richardson who first collected a specimen in May 1820 near Fort Carlton on the North Saskatchewan River, Richardson's ground squirrels (Figure. 1) are members of the squirrel family in the order Rodentia (Coues, 1875). These animals are considered to be an integral part of the grassland ecosystem and a well established component of prairie biodiversity, since archeological evidence indicates this species evolved in North America over 10,000 years ago (Michener).

2.2.1 Natural and Life History

Richardson's ground squirrels (*Spermophilus richardsonii*), commonly known as gophers, are herbivores whose habitat spans across most of the North American prairies.

2.2.1.1 Habitat and Range

Gophers are ubiquitous throughout the mixed-grasses and fescue prairies in North America, including some portions of Canada like Saskatchewan, Alberta (excluding the Rocky Mountains), and Manitoba (Coues, 1875; Michener and Koeppl, 1985). Generally speaking, gophers live in open plains with short grasses and avoid heavily forested areas (Laundre and Appel, 1986). However, they have managed to

thrive in human-modified habitats, such as grazed pastures, cultivated fields, and city parks, despite the fact that other species, such as bison and swift fox, have reached the brink of extinction on the prairies as a result of human activity (Michener and Schmutz, 2002; Downey, 2003).

Figure 2.1. Richardson's ground squirrel (*Spermophilus richardsonii*).

Photograph taken on the South Saskatchewan River bank on July, 2005.

2.2.1.2 Diet

Though relatively little is known about their natural diet, Richardson's ground squirrels are predominantly herbivores (Anderson et al., 1989). Their diets mostly consist of succulent vegetations such as forage grasses, legumes, cereal crops, pulse crops, and native grasses. Seeds of plants and domestic crops are also an important dietary supplement for gophers due to their high nutrient value and oil content, which helps in the deposition of fat necessary for hibernation (Askham, 1994). Richardson's ground squirrels may also feed on insects, such as grasshoppers, caterpillars, crickets, beetles (Leach, 1978), but they do not kill either their own species or other species for food (Michener, 1985).

2.2.1.3 Survival and Longevity

Natural mortality among Richardson's ground squirrels is quite high, particularly in males. Generally, females live four years on average, while males usually live only one year. Of those surviving to adulthood only 10 to 20 per cent are juvenile males and 40 to 50 per cent are juvenile females (Anonymous B. 2000). Three factors can contribute to the disappearance of gophers: 1) death, 2) dispersal, or 3) entry into hibernation when counting. Dorrance (1975) indicated that over winter mortality and pup loss prior to weaning are the main determinants of population size. Another possible source of mortality in gophers could be associated with fighting between adult males during early spring (Yeaton, 1972; Michener, 1979).

2.2.1.4 Population Dynamics and Sex Ratio

Gopher populations have a tendency to spike during periods of drought. In this period, pastures can be stressed by poor grass growth coupled with heavy grazing pressure from livestock and wildlife (Cooley and Jacklin, 2000). Stressed pastures or cultivated ground favor gopher survival because they are more able to sense the presence of natural predators like coyotes, hawks, and owls.

The adult sex ratio is about three or four females to one male regardless the geographic location, year, or population density (Michener, 1979; 1995). Three possible reasons to explain this include: 1) juvenile and yearling males suffer heavier predation since they disperse further than females of the same age (Sheppard, 1972), 2) the over winter mortality for juvenile males is quite high since they are still active on the surface in October and may not find suitable hibernation sites (Michener, 1972), and 3) mortality of adult males during early spring can be high due to intra-species fighting for dominance (Yeaton, 1972; Michener, 1979).

2.2.1.5 Predators and Parasites

Gophers are the favored prey of many native species to the prairies. They account for as much as 80% of the diet of aerial predators like hawks and prairie falcons (*Falco mexicanus*), as well as terrestrial predators like the long-tailed weasel (*Mustela frenata*), the North American badger (*Taxidea taxus*) and the coyote (*Canis latrans*) (Schmutz, 1988; Michener and Schmutz, 2002). Craighead and Craighead (1956) indicated that gophers are highly vulnerable to predation by raptors during the non-hibernating season. Red-tailed hawk predation alone may account for approximately a third of the loss of gopher (Luttich et al., 1970). Moreover, gophers are hosts to a large array of parasites including its major parasite Neobellieria citellivora (*Sarcophaga citellivora*), commonly known as flesh flies, and other ectoparasites such as mites, lice, fleas and ticks. Endoparasites include coccidians, which can infect gopher digestive tract, as well as trypanosomes found in the bloodstream, lymph and spinal fluid (Michener).

2.2.2 Behavior

Gophers are burrowing rodents that construct a complex net of underground channels, in which they spend majority of their life (Michener). Generally, each burrow has approximately eight entrances on average. The main entrance of a burrow network can be distinguished by a large mound of earth (Banfield, 1974).

2.2.2.1 Hibernation

Gophers have evolved to escape the prolonged winter period through hibernation, a torpor-like state where the body temperature drops while heart and respiration rates slow down dramatically (Michener, 1992). Gophers spend a long time in hibernation. For instance, adult gophers spend 7 to 9 months in hibernation, juvenile females spend 6 to 7 months, and juvenile males spend 4 to 5 months (Michener, 1998; Michener, 2002). Adult males start to hibernate in July followed by adult females (in late July to mid-August) and then emerge in March (Michener, 1995). Juvenile males, however, start hibernation in early September after juvenile females. In southern Saskatchewan, adult gophers emerge from hibernation in late March (Yeaton, 1972), whereas juveniles emerge in early June (Michener, 1973).

2.2.2.2 Daily Life Cycle

Gophers are strictly diurnal animals. Their activity during daylight allows them to easily detect predators (Canadian Wildlife Service 1979). Gophers have three peaks of activity during daylight hours: 1) about two hours after dawn, 2) 10A.M. to 2P.M., and 3) 4P.M. to sunset. They are most active on warm, sunny days, but stay under ground in excessively hot conditions or when it rains (Banfield, 1974).

2.2.2.3 Reproductive Behavior and Litter Size

Both sexes are reproductively mature at one year old. Mating occurs once a year in the spring after females emerge from hibernation. Female gophers have only one litter per year. In Saskatchewan, Richardson ground squirrels typically breed between the 7^{th} and 16^{th} of April, which is approximately one week earlier than gophers in northern Alberta (Sheppard, 1972). On average, each mature female produces a litter size of 7 neonates in Saskatchewan (Sheppard, 1972), whereas females in Alberta produce between six and nine neonates (Michener, 1995).

2.2.3 Biological Role and Public Perception

Gophers are an essential part of the ecosystem on the prairies (Leach, 1978). However, western farmers also consider gophers to be serious burden dating back to as early as 1894 (Banfield, 1974).

2.2.3.1 Positive Impact

Gophers are an important component of the prairie ecosystem. They play an essential role in the formation and areolation of the soil through the construction of burrows, which has been found to increase plant and animal diversity in related areas (Leach, 1978). Also, gophers contribute to the fertility of the soil through the addition of organic material from excretions and their carcasses (Banfield, 1974).

Additionally, gophers are the primary source of food for many native predatory species on the prairies. They account for up to 80% of the diet of some raptors such as Swainson's hawks (*Buteo swainsoni*), ferruginous hawks (*Buteo regalis*), prairie falcons (*Falco mexicanus*), and red-tailed hawks (*Buteo jamaicensis*) as well as terrestrial predators like long-tailed weasels (*Mustela frenata*), red foxes (*Vulpes vulpes*), and coyotes (*Canis latrans*) (Michener). During the nestling period gophers average 89% of the total prey for ferruginous hawks and 69% for the other raptors (Schmutz, 1988). For example, in Alberta, gophers are the primary prey species (75% of biomass) of Swainson's hawks during the nestling period (Schmutz, 1988).

2.2.3.2 Negative Impact

Richardson's ground squirrels have long been considered agricultural pests throughout the prairies (Brown and Roy 1943), because they pose a great economic burden on the farmer. As such, gopher population control is related to the agricultural activities aimed at the reduction of economic losses and health hazards to both humans and farm animals.

Crop damage most often occurs when gophers enter the edges of the crop area from their residence in the uncultivated border areas (Sullins and Sullivan, 2000).

Gophers can cause damage by consuming and trampling the crops. A single gopher may consume up to a bushel of grain per year (Banfield, 1974). Thus, damage to cereal crops is quite severe on the edges of fields adjacent to native grasslands (personal observation).

Being fossorial animals, gophers destroy pastures, golf courses, as well as some underground equipment such as pipes, conduits, and cables (Figure 2.2 and Figure 2.3). Furthermore, mounds of soil excavated from their burrows can smother desired vegetation, which reduces crop yields and the vegetation for livestock (Rulofson, 1993; Miller et al., 1994). The excavations may also cause damage to machinery used in the harvest of forage crops (Anonymous C). Also, gopher burrows can cause water loss in irrigated fields (Rulofson, 1993).

Although no scientific data is available regarding the health hazards of gopher burrows to farm animals, testimony from farmers indicates that cows and horses can step into the burrows and break their legs (Champoux, 2005). Furthermore, gophers pose a public health concern, since they are hosts to at least two species of fleas that transmit the bubonic plague (Leighton, 2001) and are also reservoirs of tularemia and Rocky Mountain spotted fever (Banfield, 1974).

Figure 2.2. Gopher's burrow next to the sidewalk, which can be hazardous to pedestrians and can be costly to repair.

Figure 2.3. Gophers make burrows under the light posts, which can damage circuitry potentially resulting in traffic problems.

2.3 Traditional Control Measures

Gophers can invade crop fields to establish complex burrow systems, thus, forage loss can be caused by both burrowing and feeding. Two methods of gopher control are available to reduce gopher numbers: 1) chemical and 2) non-chemical (physical) methods.

2.3.1 Physical Methods

Physical methods such as shooting and trapping have been used to control gopher population for centuries. However, these methods are only effective on very small "hot spot" areas and are very labor-intensive.

2.3.2 Chemical Methods

Poisoning with a treated grain is the most common method used on farms and ranches. Strychnine, zinc phosphide and anticoagulants are the most commonly used poisons for gopher control.

2.3.2.1 Commonly Used Toxins and Their Mechanisms

Strychnine: The LD_{50} of strychnine for most species ranges from 2-25mg/kg (Hudson et al., 1984), with rodents typically in the range of 5-6 mg/kg (Prakash, 1988; Mutze, 1989). Strychnine is a stable alkaloid that retains its toxicity indefinitely in bait as well as in carcasses of poisoned animals. When strychnine enters the bloodstream, it causes a coordinated extensor thrust and tetanic convulsions with death resulting from prolonged paralysis of respiratory muscles (Philippe et al., 2004). Typical signs of strychnine poisoning are restlessness and muscular twitching, which progresses into convulsive seizures continuing for 45 minutes or more before death (Osweiler et al., 1985). Poisoned animals often die in less than an hour, but death can take 24 hours or longer when sub-lethal doses are ingested. This indicates that animals poisoned by strychnine might suffer a long time before the eventual death. Strychnine is one of the most toxic compounds. It can be lethal to almost all vertebrate species including human, domestic animals and wildlife (Hegdal and Gatz, 1977).

Zinc phosphide: Zinc phosphide is a popular rodenticide due to its low cost and rapid onset of action. It is a non-specific pesticide used for vertebrates. Zinc phosphide reduces to phosphine gas under acidic aqueous conditions (like in the stomach of rodents). Phosphine gas causes central nervous system depression, lung irritation, and liver and other organ damage (Land Protection, 2002). Death is usually the result of heart failure and anoxia (lack of oxygen in tissues).

Zinc phosphide bait poses a low hazard to non-target animals in terms of secondary toxicity. Baits containing zinc phosphide are not likely to be consumed when sufficient food is available. Furthermore, the garlic odor of zinc phosphide reinforces bait shyness (Land Protection, 2002). Another possible reason is that most animals that feed on rodents are unaffected because this rodenticide does not accumulate in the muscles and tissues (Johnson and Fagerstone, 1992). Although most predators discard the viscera, toxic effects do occur in predators due to ingestion of a poisoned animal's digestive tract (Johnson and Fagerstone, 1992).

Anticoagulants: Anticoagulant poisons are classified by their different characteristics into two generations: 1) old generation, which includes compounds such as Warfarin, Coumatetralyl, Pival, Chlorophacinone and Diphacinone, and 2) second generation, which includes Difenacoum, Bromadiolone and Brodifacoum (Lund, 1985). Anticoagulants constitute a large group of toxins that affect the blood clotting cascade. They are commonly used for rodent population control and some of them are used in the treatment of human conditions associated with thrombosis. However, rodents are much more susceptible to anticoagulant poisoning than other animals and humans (Anonymous B, 2000).

Regardless of their classification all anticoagulants act in a similar fashion. They inhibit the generation of the active form of vitamin K_1 via the inhibition of vitamin K_1 reductase. Activation of clotting factors II, VII, IX, and X requires the presence of vitamin K as a cofactor. When vitamin K cannot be regenerated, clotting factors are not activated, which will result in a coagulopathy involving both the extrinsic and intrinsic pathways (Burkhart, 2000).

First and second generation anticoagulants are widely used to control rodents. However, a problem remains with primary and secondary toxicity to non-target species. For example, secondary toxicity of anticoagulants in barn owls causes sub-lethal haemorrhaging for several days (Godfrey, 1985). As a result, ethical and animal welfare concerns have increased with regard to both target and non-target animals.

2.3.2.2 Bait Delivery

The best time for bait delivery is during the breeding period, i.e. two or three weeks after emergence, but before vegetative growth has begun, when both sexes are active. Because the entire population is active, animals may accept baited grain more readily (Sullins and Sullivan, 2000). Control at this time eliminates adults and potential young, and ensures minimal crop damage by the rodents for the year.

There are two commonly used methods of bait delivery: 1) hand baiting, and 2) bait station. Hand baiting at each burrow is labor intensive and expensive, while bait stations can increase both the effectiveness and safety of the baits. One bait station will expose rodents from 50 up to 100 m away each station should then contain about 500 g of bait and be checked daily to maintain an uninterrupted supply of bait for a few weeks or until feeding ceases.

2.4 Current Approaches to Control Gophers and Their Limitations

Currently, chemical controls are the main gopher control methods on the prairies. However, gopher control (according to the recent survey from Saskatchewan) with toxin baits in crop fields is often perceived as ineffective in reducing population densities. This may be due to several factors such as poor bait acceptance, sub-lethal dosing, social feeding, and dietary preferences, which reduces the efficiency of rodenticides (Eisemann et al., 2003).

2.4.1 Strychnine

Strychnine, distributed on oats, has been reported as an effective gopher control method. This method reduced gopher populations by more than 70% in the past

(Sullins, 1984). However, a recent study conducted by McKinnon (2004) showed that only 53% reduction in gopher numbers when strychnine was distributed on wheat. The major disadvantage of strychnine is its lethalness to most vertebrates including humans (Lindsey et al., 2004), domestic animals, wildlife (Hegdal and Gatz, 1977), and other non-target animals. As a result, strychnine has been banned from use in New Zealand (Eason and Wickstrom, 2001).

2.4.2 Zinc Phosphide

Zinc phosphide is a less expensive alterative to strychnine. It is an effective acute rodenticide used for gopher control (Matschke et al., 1983; Sullins & Sullivan, 1995). Under ideal circumstances, zinc phosphide can be 85% to 90% effective (Askham, 1994). However, its use is limited due to its instability and garlic like odor. Therefore, when rodents only consume a sub-lethal amount of the poison and become ill, they associate their illness to the food most recently consumed (i.e. the bait). This leads to the refusal to eat the bait again (i.e. bait shyness) (Shumake et al., 2002). The strong, garlic like odor emanating from zinc phosphide baits also make this compound readily identifiable, which reinforces bait shyness (Jackson, 2001).

2.4.3 Anticoagulants

The first generation of anticoagulants such as warfarin and its derivatives dominated rodent control for many years. However, these agents encountered the problem of resistance (Boyle, 1969). Consequently, second generation anticoagulants were developed and designed with a higher acute toxicity to counter this resistance.

Second generation anticoagulant compounds have been used successfully in recent years (Lund, 1985). They do not cause bait shyness and rodents have a greater susceptibility to anticoagulant than non-target species such as birds (Bentley, 1972). Moreover, a powerful antidote (Vitamin K1) is available to treat accidental acute toxicity. Nevertheless, the continued extensive use of second-generation anticoagulants poses a significant hazard to non-target species through sub-lethal or lethal poisoning resulting from secondary exposure (Eason et al., 2002). Residues of brodifacoum (a

second generation anticoagulant) have been detected in a range of native New Zealand birds and introduced mammals (Bailey et al., 2005). In addition, this type of rodenticide has also encountered resistance problems especially in populations largely resistant to the first generation of anticoagulants (Gill et al., 1992).

2.5 Bait Shyness and Pesticides Resistance

One of the important causes of pesticide resistance is bait shyness. Bait shyness refers to the consumption of a sub-lethal amount of the poison causing illness and the association of their illness with the food most recently consumed (i.e. the bait). This can eventually lead to the refusal for future consumption of the baits (Shumake et al., 2002). For example, rats employ cautious (or neo-phobic) feeding strategies (Kandel and Chenoweth, 1952) and acquire an associative memory that allows them to develop learned aversions. As a result they can detect toxins in food and regulate their intake to sub-lethal levels, a mechanism termed "stopfeeding" (Dean and Cook, 1996).

Resistance to pesticides is almost an inevitable development among pest species (NRC, 1986). For example, when pests are exposed to an increased dose of pesticides, they develop a higher resistance to the toxin, which may then be passed on to offspring as a heritable trait (NRC, 1986). This gradually results in an increasing percent of pest population becoming resistant to control products that originally worked well.

The evolution of pesticide resistance amongst rodents is common in pest populations (Brattsten and Ahmad, 1986). Resistance occurring in response to anticoagulant baits used in rodent control is a good example (Greaves, 1985). When resistance develops with repeated anticoagulant use, a noticeable increase in the severity of the infestation occurs (Greaves, 1985). Such resistance can be transferred to the offspring, and consequently anticoagulant efficacy may be limited. The first substantiated report of this phenomenon was described in 1958 in Norway rats treated with warfarin (Boyle, 1969). Since then, this phenomenon has now been widely recorded in North America and Europe (Meehan, 1984). Furthermore, it has also been found that warfarin-resistant rodent strains often possess cross resistance to many other first generation anticoagulant compounds. What is even more problematic, the

mechanisms of anticoagulant resistance are still not completely understood (MacNicoll, 1986). Different mechanisms of anticoagulant resistance have been described in rodents (MacNicoll, 1993). In this case, a more specific pesticide development based on the resistance mechanisms to deal with the resistance population will be more complex.

Currently the standard economic conceptualization of resistance regarding susceptibility to a pesticide or classes of pesticides is seen as an exhaustible resource that is gradually depleted through pesticide application (Hueth and Regev, 1974). The response to pesticide resistance traditionally has been to apply a larger amount of pesticide, to increase the frequency of application, or to interchange pesticides used. The first two solutions are self-defeating, as they tend to exacerbate the development of resistance. The third solution may ultimately cause its own resistance problems, since there is no basic change in the mode of action and treatment strategy of pesticide use (Roush, 1981). Currently, for rodent control the best way to avoid resistance is to periodically rotate baiting with non-anticoagulant bait (often an acute poison that will kill any rodents demonstrating resistance to anticoagulant baits).

Recent scientific advances have made progress toward combating the development of resistance in pests. The existing pesticides can employ several strategies useful to overcoming the problem of resistance. An example of this is pesticide mixtures including different modes of action for pesticide rotation. Consequently, the possible development of resistance is one of the most important considerations during new pesticide development. In order to avoid possible problems of resistance, a better understanding of the biochemistry and genetics of resistance development is necessary.

2.6 Animal Welfare Issues

Pest control measures kill millions of rodents annually just because they are perceived as pests. However, very little attention has been paid to the humaneness of pest control methods. Taking the methods acceptable for pets into consideration, one would expect currently used methods to control rodent populations to be unacceptable. The general perception is that animal welfare issues do not apply to pests. Yet, the physiological aspects of pain and suffering are as important in pest animals as they are in

other animals (Mason and Littin, 2003). The most widely accepted methods of gopher control are chemical poisonings, which can cause much pain and suffering in target and non-target species. This bespeaks to the need to develop management practices of gopher control that address welfare concerns for both target and non-target animals.

2.6.1 Target Animals: Gophers

The most popular rodenticides used for gopher control are chemicals such as strychnine, zinc phosphide and anticoagulants. These compounds have several common welfare issues. First, the death of nursing adult females will lead to death of any dependent pups in the nest due to dehydration and starvation. Second, these compounds can cause considerable suffering in the target animals as well as non-target animals. For example, anticoagulants can cause external bleeding, pale extremities, accompanied by bloody diarrhea. Also, they can cause some internal injuries like multiple hemorrhages throughout muscles and the intestinal tract (Eason and Wickstrom, 2001). Strychnine can cause convulsions, which are extremely painful because of the rupture of tendons and joint capsules (Philippe et. al., 2004).

2.6.2 Non-target Animals: Primary and Secondary Toxicity

The basic intent of pest control is to design pesticides that are highly toxic to pest species but provide a useful margin of safety to non-target species. However, since the definition of a pest depends not on taxonomy or physiology but rather on its ecological role, a conflict exists with other species that share many biochemical pathways, physiological functions, anatomical features, and life history attributes with non-target species (NRC, 2000). As a result, the use of poison to control unwanted wildlife poses potential threats to predators and other non-target species (Mendenhall and Pank, 1980). This complicates pesticide design that contains specific desired activity against the target species and is safe to all other non-target species. Consequently risk to non-target species from a compound is determined by their intrinsic susceptibility to properties of the poisons used (i.e. the toxicokinetics of these chemicals), the bait design, and the way in toxic baits are used in the field.

Strychnine, which is toxic to most species (Hudson et al., 1984) and has been used for a long time to control gopher populations, poses both primary and secondary potential threats to non-target animals. For example, Swainson's hawks and other raptors may be subject to secondary poisoning from scavenged or dying gophers that are poisoned by strychnine (Schmutz et al., 1989). Despite the fact that raptors eviscerate gophers prior to consumption, which will reduce the rate of secondary poisoning, eviscerated squirrels sometimes still contain poisoned bait in their cheek pouches which can cause secondary poisoning. Barn owls have been identified to hold sub-lethal haemorrhaging for several days after eating intoxicated animals (Godfrey, 1985). On the other hand, mammalian predators may consume the poisoned gopher's viscera and face even greater danger from secondary poisoning than hawks (Schmutz et al., 1989).

2.7 Toxin Metabolism

Ingestion provides the most convenient and practical means of toxin delivery to control wild animals. The ingested toxins first encounter the intestinal wall and liver where they undergo metabolism before they enter systemic circulation and other organs and cause systemic toxicity. The basic purpose of xenobiotic metabolism is to make compounds more water soluble and, thus, more readily excreted in the urine or bile. The toxic effects elicited by a chemical may be influenced by its biotransformation, either activity is diminished, completely alleviated, or the chemical is transformed into a more toxic metabolite. Consequently, biotransformation may affect the duration and the intensity of toxic effects, as well as the pattern and the amount of residues that is eventually left in the environment (Boobis, 1992).

2.7.1 Hepatic Cytochrome P450 System

Xenobiotic metabolism occurs through two phases of biotransformation: Phase I and Phase II. Phase I enzymes are responsible for the initial changes in the molecular structure of the toxin, followed by conjugation with specific endogenous molecules catalyzed by Phase II enzymes. Over 90% of Phase I metabolism is mediated by the cytochrome P450 system (Lewis et al., 1998). Furthermore, in mammals toxin metabolism occurs primarily in the liver, which is also the major site of cytochrome

P450 proteins (Lewis et al., 1998).

The general structure of cytochrome P450 is a low spin ferric haemoprotein with a thiol residue as an axial haem ligand that contains a C-terminal rich with helixes and N-terminal enclosing beta sheets (Poulos and Raag, 1992). Its name is derived from the strong absorbance at 450nm when reduced with carbon monoxide (Wheeler and Guenthner, 1990). Cytochrome P450 enzymes play an important role in the metabolism of a wide variety of both exogenous and endogenous substrates (Degtyarenko and Archakov, 1993).

Cytochrome P450 isozymes use the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH) and atmospheric oxygen, whereby one atom of molecular oxygen is inserted into the chemical with the other oxygen atom being simultaneously reduced to water. Reactions catalyzed by these enzymes include N-oxidation, S-oxidation, dealkyation, deamination, and the removal of hydrogen and sulphur groups. Although, these enzymes are most abundant in the liver microsomes (small particle consisting primarily of fragmented endoplasmic reticulum), they can also be found in the intestines, lungs, and other organs (Philpot, 1991; Kolars et al., 1994).

Of note, the cytochrome P450 system can be regarded as a double-edged sword; the enzymes are capable of both activation and de-toxication of chemicals. Some of the isoforms are able to mediate the activation of certain compounds and others are largely associated with detoxifying metabolism (Guengerich, 1997; Omura, 1993).

2.7.2 Differences in toxin metabolism

The significant inter-individual differences in the response to xenobiotics are principally due to the differences in capacity for toxin metabolism caused by genetic polymorphisms or by inhibition or induction of toxin metabolism. One of the most important aspects of cytochrome P450 enzyme system is that the metabolic capacity of the cytochrome P450 enzyme system is not equal in all members of a population, and as a result, the metabolic conversion and excretion rate of toxins varies among individuals ranging from extremely slow to ultra fast (McKinnon and Evans, 2000). There is no

scientific data available on xenobiotic metabolism in gophers.

Inter-individual variability in metabolic rate is largely determined by genetic factors. Mutant alleles carrying certain nucleotide substitutions, deletions, insertions or gene conversions, may lead to variance in enzyme activity, which results in differences in toxin metabolism and toxic outcomes and/or response. In addition, environmental factors such as diet and pollutants can also cause changes in cytochrome P450 expression level of different individuals, which can lead to variations in toxin metabolism.

2.7.2.1 Genetic Polymorphism

Genetic polymorphisms are common variations in the genetic code, typically defined as comprising at least 1% of the population or sample of interest. They may also be linked to inherited autosomal recessive traits (Poulsen and Loft, 1992). Biologically, it is very important for species to hove genetic polymorphisms. It provides an organizational arrangement whereby variability is structured into a population so that it has a better chance of survival should changes occur in the environment (Smith, 1986). Genetic polymorphisms are important contributors to the inter-individual differences in xenobiotic biotransformation within a population. Numerous specific genetic polymorphisms in cytochromes P450 super-family in humans have been described in past decades (Engel et al., 1996; Cytochrome P450 homepage).

2.7.2.1.1 Types of Genetic Polymorphism

Variability in cytochrome P450 genes may be broadly divided into two types, structural and regulatory (McKinnon and Evans, 2000). A polymorphism identified in the coding region is termed a structural polymorphism (McKinnon and Evans, 2000). The coding region of a gene determines the amino acid sequence of the encoded enzyme and the variability of amino acids has the potential to alter enzyme activity. Alternatively, individual xenobiotic metabolism capacity can be determined by the variability in regulatory regions of cytochrome P450 genes that can be termed a regulatory polymorphism (Boobis, 1992). Regulatory regions of a gene are generally

non-coding sequences that are involved in regulating protein levels. Genetic variability in these regions is associated with altered levels of protein rather than changes in the protein itself.

2.7.2.1.2 Inter-species Differences

Generally, the basic reactions and major metabolites of a xenobiotic are similar among species. However, small differences in metabolism can result in major differences in susceptibility to toxicity. Various mechanisms can be associated with differences between species in response to a xenobiotic. These include: 1) lack of, or genetic defect in a particular metabolic pathway, 2) differences in the K_m and V_{max} of specific enzyme, 3) the existence of different isozymes, 4) differences in the ratios of important specific isozymes, and 5) differences in the ratio of activities of separate enzyme systems that act together to metabolize a specific xenobiotic (DeBethizy and Hayes, 1989). Thus, it is possible to trace species differences in xenobiotic metabolism to variations in cytochrome P450 levels, their inducibilities, and the existence of different cytochrome P450 isoforms of the same protein family or subfamily in various species (Lewis et al., 1998).

2.7.2.1.3 Intra-species Differences

Notable age, sex and strain differences in xenobiotic biotransformation with respect to activation or detoxification are observed within the same rodent species (Schenkman and Greim, 1993; Nakajima et al., 1992).

2.7.2.1.3.1 Sex Dependent Difference

Sex dependent differences in hepatic xenobiotic metabolism occur in fish, birds, and mammals (Pampori and Shapiro, 1999). However, rodents exhibit the most pronounced sexual dimorphism in metabolism (Kedderis and Mugford, 1998). The extensive variations in metabolism by rats may be the result of extensive inbreeding or differential evolution of cytochrome P450 isoforms in mammals, which can be attributed to the expression of sex-specific cytochrome P450 enzymes. Normally, male rats have

higher rates of xenobiotic metabolism than females (Kedderis and Mugford, 1998). For example, studies have shown that the predominant male isoforms are CYP2A2, CYP3A2, CYP2A1 and CYP 2C11, whereas female express CYP2C12 and 3 to 4 times greater levels of CYP2C7 than males (Pampori and Shapiro, 1999).

Sexual dimorphisms are produced by either imprinting or activational factors, or often by a combination of the two (Kedderis and Mugford, 1998). The expression of some isoforms of hepatic cytochrome P450 enzymes in adulthood is programmed in the perinatal period of life, which is a type of regulation termed enzyme imprinting (for details, see 2.7.4). Studies showed that the level of testicular androgens in the blood irreversibly imprint the liver to express specific cytochrome P450 isoforms in the adult rats through neonatal imprinting (Gustafsson et al., 1983). In contrast, females are not as dependent on circulating levels of estrogens for the expression of female cytochrome P450 isoforms as males are for the expression of male isoforms of cytochrome P450 isoforms from androgens (Kedderis and Mugford, 1998). Patterns of growth hormone secretion (Mode et al., 1982; McClellan-Green, et al., 1989), somatostatin (Kobliakov et al., 1991), insulin, and thyroxin (Peng and Coon, 1998) also play specific roles in the sex-specific expression of CYP450 isoforms in rats. Furthermore, these sex dependent differences have also been observed in mice (MacLeod et al., 1987) and beagle (Lin et al., 1996). However, a comprehensive understanding of the mechanisms responsible for the sex-specific expression of the microsomal cytochrome P450 is still lacking.

2.7.2.1.3.2 Age Dependent Differences

Generally, the level of hepatic microsomal monooxygenase activity (cytochrome P450 activity) is very low during fetal development in most mammals, but increases rapidly soon after birth (Nakajima, 1992). For example, human infants do not develop a mature enzyme system until one year of age (Alcorn and McNamara, 2002a,b). However, elderly have age related decreases in liver mass, hepatic enzyme activity, and hepatic blood flow, which can result in the reduction of overall metabolic capacity of the liver (Loi and Vestal, 1988).

Hepatic xenobiotic biotransformation involving cytochrome P450 enzyme

system can be influenced by both sex and age at the same time. For expample, sex dependent differences in cytochrome P450 content and monooxygenase activities disappear as rat age (Kamataki et al., 1985). Young male rats exhibit greater enzyme activity levels than females. However, with age, males became similar to females with respect to enzyme activities (Kamataki et al., 1985). Changes in the pattern of growth hormone secretion may explain these changes.

2.7.3 Non-Genetic factors

Although individual metabolic capacity is determined predominantly by genetic background, several internal and environmental factors additionally influence the activity of cytochrome P450 enzymes. Diet and certain diseases with hepatic involvement may affect the levels and activities of cytochrome P450 isozymes by different mechanisms. For example, drugs used to treat diseases can affect levels of cytochrome P450 isoform by interfering at any stage of cytochrome P450 enzyme synthesis process. These drugs can change: 1) the rate of degradation of specific mRNA, 2) transcription of specific cytochrome P450 genes, 3) the translation process, and 4) enzyme degradation through protein turnover or by suicide inhibition. Substrates of the cytochrome P450 can also directly affect the system itself. Cytochrome P450 substrates or their metabolites can act as inducers or inhibitors for this system by binding to the main components, by affecting the interaction between these components, or by affecting key steps in the catalytic cycle, which causes change in the activity of the system (Yang et al. 1992).

2.7.4 Neonatal Cytochrome P450 Imprinting and Inherited Individual Variation in Toxin Response

Imprinting involves the inheritance of a silenced allele of a gene through either or both a paternal or maternal germ line. Imprinted genes are unusual autosomal as they are expressed predominantly from either the paternal or maternal inherited alleles in somatic cells (Haig, 2000). However, since imprinting persists in inbred genetically identical mice this suggests that the imprint is solely an epigenetic phenomenon.

Furthermore, evidence suggests that imprinting is controlled at the level of transcription (Burns et al., 2001).

Cytochrome P450 imprinting plays an important role in sex-dependent and age-dependent differences in toxin metabolism. Sex-dependent rat hepatic cytochrome P450 enzymes have been identified. Research suggests that these enzymes are regulated mainly by the sex-specific profiles of circulating growth hormone, and are subject to androgen imprinting (Chang, et al., 1996). For example, Pampori and Shapiro (1994) suggested that depending upon neonatal exposure levels, monosodium glutamate can produce permanent, yet very different defects in the adult male patterns of growth hormone secretion and growth hormone-dependent CYP2C11 (male-specific hepatic cytochrome P450 isoform) expression. Kawai et al. (1999) identified that treatment of neonatal rats with tamoxifen produced a long-lasting effect on hepatic CYP2A1, CYP2C11, and CYP3A9 expression. Ishizuka et al. (2003) suggested that the developmental induction of CYP2C11 is imprinted by exposure to testosterone-derived estrogen during the neonatal period.

2.7.5 Induction and Inhibition of Cytochrome P450 System

Two of the most common causes of altered xenobiotic biotransformation reactions are induction and inhibition of cytochrome P450 enzymes (Meyer and Rodvold, 1996). Inhibition of cytochrome P450 enzymes can be classified generally into reversible inhibition and irreversible inhibition based on the enzymatic mechanism (Yan and Caldwell, 2001). Reversible inhibition, including competitive and noncompetitive inhibition, is the most common mechanism. Competitive inhibition occurs when the inhibitor binds to the same site on the enzyme as the substrate. Noncompetitive inhibition occurs when the inhibitor binds to another site on the enzyme. Many of the potent reversible cytochrome P450 inhibitors are nitrogen-containing chemicals, including imidazoles, pyridines and quinolines (Lin and Lu, 1998). Antifungal imidazole derivatives such as clotrimazole (CTZ) and sulconazole are generally recognized as potent competitive inhibitors of cytochrome P450 enzymes since they are potent ligands of the heme iron atom (Olkowski et al., 1998; Zhang et. al.,

2002).

In contrast to reversible inhibition, where noncovalent interactions govern inhibition binding to the enzyme, irreversible inhibition (mechanism based inhibition) occurs when certain compounds are metabolized by the cytochrome P450 system to active metabolites that covalently bind to the enzyme and cause an irreversible loss of function (Lin and Lu, 1998). Activity under this circumstance can only be restored through synthesis of new enzymes, a process that requires several days.

Irreversible inhibition falls under two categories. The first type involves the formation of metabolic intermediate complexes. These metabolites then bind tightly to the prosthetic haem iron of cytochrome P450 enzymes forming stable complexes. This can lead to the reduction of cytochrome P450 enzyme activity, or complete deactivation or loss of the enzyme activity. The second type is caused by covalent modifications of cytochrome P450 enzymes by reactive intermediates, which are found either at the haem group or on cytochrome P450 proteins. In general, this type of irreversible inhibition modification of the haem group invariably inactivates the cytochrome P450 enzyme, since the haem group is the oxidation center of cytochrome P450 enzymes (Yan and Caldwell, 2001).

An important aspect of the cytochrome P450 enzymes is that some, but not all, are inducible. With induction, an increased activity is not due to activation of the normal enzyme level, but rather is related to a complex process involving new RNA and the synthesis of more enzyme protein (Bresnick, 1978), which enhances the enzyme's metabolizing capacity (Dossing, 1983). Thus, compared to inhibition, which is an almost immediate response, cytochrome P450 induction is a slower regulatory process.

2.7.5.1 Practical Implications

Cytochrome P450 enzyme inhibition can result in three consequences: 1) an increase of toxicity caused by decreased toxin metabolism if the parent compounds are more toxic, 2) a decrease of toxicity through decreased formation of reactive metabolites if the metabolites are more toxic, and 3) the interaction between compounds may occur,

which can increase or decrease another compound's toxicity, if the co-administered compound is a substrate for the same cytochrome P450 enzyme. Usually metabolites are less toxic than the parent compounds; therefore, induction is an adaptive response that protects the cells from toxic xenobiotics by increasing the detoxification activity. Nevertheless, the induction of cytochrome P450s associated with metabolic activation should also be regarded as indicative of potential toxicity (Ioannides and Parke, 1993).

2.8 Summary of Pertinent Facts

There is a tremendous need for compounds that will control pests (such as gophers) more efficiently and more specifically. New pesticides have to be efficacious and environmentally acceptable. In the development of a new pesticide, the use of both *in vitro* and *in vivo* models is essential for the successful outcomes. However, *in vivo* experiments have some associated problems regarding their ethics and financial viability. *In vitro* studies with experimental models offer an alternative that may help in the understanding of the metabolic processes responsible for toxin metabolism in the gopher. Knowledge from the *in-vitro* systems can then be extrapolated to *in vivo* situations where the comparison of non-target species with gophers may lead to the development of an experimental model that can be used in practice. These *in vitro* systems include miniaturized systems and mechanism-based screens in either modified microorganisms or *in vitro* enzyme assays, with a particular molecular or enzymatic target. In our study, the molecular targets selected are particular to gophers, which are not found in non-target organisms. This type of investigation can be restricted to compounds that will selectively control gopher populations with less chance of non-target toxicity. Furthermore, pesticides with new mechanisms of action have become more valuable due to the potential that mechanism-based screens can find compounds that hit new target sites (cytochrome P450 enzymes), where there is no known resistance.

In conclusion, understanding of toxin metabolism may help in the development of better means of toxicological control. In particular, the knowledge of metabolic weaknesses may help in the design of more effective, more humane, and target species

specific toxins. However, the restriction is the discrepancy between the activity detected with *in vitro* assays (microsomal metabolism) and the *in vivo* activity. Therefore, the development of novel means of toxicological control of pests requires extensive experimentation.

3.0 EVALUATION OF VARIOUS COMPONENTS OF HEPATIC CYTOCHROME P450 SYSTEM

3.1 Introduction

Richardson ground squirrels (*Spermophilus richardsonii*), more commonly known as gophers, are ubiquitous throughout the mixed-grass and fescue prairies in North America (Coues, 1875; Michener and Koeppl, 1985). Gophers adapted very well to human-modified habitats, such as grazed pastures and cultivated fields (Michener and Schmutz, 2002; Downey, 2003), and they became a serious burden to prairie farmers as early as 1894 (Banfield, 1974). Destroyed crops were a fact of life for hundreds of settlers (Anonymous A, 1919).

Even though gophers play an important role in the ecosystem (Leach, 1978; Schmutz, 1988), they are principally considered a major pest (Brown and Roy, 1943; Michener, 1995). Gophers cause significant economic losses as a result of crop destruction (Michener; Banfield, 1974; Andelt and Race, 1994), competition for forage with farm animals (Rulofson, 1993; Miller et al., 1994), equipment damage, and irrigated field water loss (Rulofson, 1993). Gophers also present a health hazard to pastoral animals and humans. No scientific data is available on the health hazards to farm animals due to gopher burrows; however, farmers' testimony indicated that cows and horses can step into burrows and break their legs (Champoux, 2005). Furthermore, gophers may carry fleas, which are vectors for the bubonic plague (Leighton, 2001) and a reservoir of tularemia and Rocky Mountain spotted fever (Banfield, 1974).

The attempts to control gopher populations commenced more then a century ago (Coues, 1875; Canada Department of Agriculture, 1916; Canadian Wildlife Service, 1979). Historically, rodenticides such as strychnine, zinc phosphide, and anticoagulants were used as the main methods of gopher control (Matschke et al., 1983; Schmutz et al.,

1989; Farm Facts). However, these chemical means of control appear ineffective. Interestingly, a survey conducted in 2001 in Saskatchewan suggests that the problem of gophers has increased over the last ten years (2001 Saskatchewan "Gopher" Survey).

Understanding the dynamics of metabolic processes responsible for toxin metabolism in gophers may help to explain the failed attempts to control these animals using existing poisons. Accordingly, the objectives of the present study were to evaluate the detoxifying potential of gophers by examining the hepatic cytochrome P450 enzyme system. The experimental approach for this study was based on 2x2 factorial designs with the main factors being: 1) gender (male versus female gophers); and 2) status (gophers previously exposed to toxin versus naïve gophers).

Because the liver is the major site of xenobiotic biotransformation, it is the tissue of choice for metabolism and toxicity studies. Gopher liver microsomal fractions were used as an *in vitro* model to evaluate the activity of the cytochrome P450 system (CYP450), which is the major enzyme system responsible for detoxification. The measurements included: 1) CYP450 content, 2) Cytochrome b5 content, and 3) NADPH-Cytochrome P450 reductase activity. All three components are located on the endoplasmic reticulum (ER). Cytochrome P450 is the terminal oxidase of the whole system. Cytochrome b5 is a membrane bound protein, which can donate the second but not the first electron to CYP450. Thus, cytochrome b5 may affect CYP450 enzyme-mediated xenobiotic metabolism by shunting electrons either toward or away from CYP450 (DeBethizy and Hayes, 1989). As well, cytochrome b5 enhances the coupling of NADPH oxidation to substrate hydroxylation (Waskell et al., 1991). NADPH-cytochrome P450 reductase, a flavoprotein, contains both FMN and FAD, catalyzes the transfer of electrons from NADPH to CYP450 (Dignam and Strobel, 1975). These components work together and play a key role in the catalytic cycle in xenobiotic metabolism.

3.2 Materials and Methods

3.2.1 Chemicals

Potassium phosphate, ethylenediamine tetraacetic acid (EDTA), β-NADH, and dithionate were obtained from Sigma (Sigma, St Louis, Missouri, USA), Advanced Protein Assay kit was obtained from Fluka (Switzerland).

3.2.2 Animals

Gophers were trapped from various locations in central Saskatchewan. Live traps (Tomahawk Live Trap Co. PO Box 323, Tomahawk, WI 54487) were provided by the Department of Biology. All procedures and contingency plans for this project were prepared in consultation with Dr. Francois Messier from the department of Biology who has extensive experience in research involving trapping of animals.

Gophers were harvested from two different sites. The first site (A) was selected on University of Saskatchewan fields located near the South Saskatchewan River. This site was severely infested with gophers. Based on our count, the area covering approximately 1 hectare was inhabited by 75 to 100 gophers. No documented poison was used on this location to control the gopher population; hence, this site provided naïve animals for our study. The other site (B) was selected on University of Saskatchewan Goodale Farm pasture. Based on the testimony of the management, regular attempts to control gophers were made on this site using baits laced with strychnine over the last several years.

The trapping of gophers on Site A was carried out between the 6th and 8th of July, 2004, and on Site B, between the 12th and 15th of July, 2004. A total of 18 gophers were trapped on site A, and 19 on site B. The trapped gophers were moved to the Animal and Poultry Science Laboratory for further processing.

3.2.3 Anesthesia and Surgery

For organ retrieval procedures the animals were anaesthetized using the inhalation anaesthetic, isoflurane. Induction of anaesthesia was accomplished with an isoflurane concentration of 3% delivered from an agent specific precision vaporizer with an oxygen flow rate of 1.2 L/min. This was delivered via a face mask and a Bain anaesthetic breathing circuit. Following induction, a state of surgical anaesthesia was maintained with 4% isoflurane and oxygen flow of 1 L/min. A plane of anaesthesia suitable for surgery was usually achieved within 2 to 3 minutes. Once the animals were in a state of deep anesthesia, a mid-line incision along the abdominal and thoracic inlet was made. The gastrointestinal tract was retracted caudally to allow for visual orientation of the liver. In order to retrieve the organ, the sternum was gently lifted, the diaphragm was opened, and the entire liver was excised using blunt-ended scissors. Following this, the animal was euthanatized and liver tissues were harvested immediately and processed at 4^{o}C.

3.2.4 Liver microsomes preparation

Liver microsomes were prepared as described by Guengerich (1989) with minor modifications. A total of 24 animals were randomly chosen for the microsomes preparation.

The livers were removed, immediately weighed, and placed in ice-cold buffer A (1.15%KCL, 1mM EDTA, pH7.4). The tissue was rinsed thoroughly and immediately perfused with ice cold buffer A. The perfusion was carried out until the perfusion fluid exiting the liver tissue was clearly free of blood (Figure 3.1). This procedure greatly enhanced the purity of microsomal preparations. The perfused tissue was cut into smaller pieces, and snap-frozen in liquid nitrogen. The frozen samples were stored at -70^{o}C until further processing.

Frozen samples were thawed, cut into thin slices, and blot dried. The slices were weighed, minced with scissors and transferred to plastic centrifuge tubes to which buffer B (1.15% KCl, 0.05M Tris, 1mM EDTA, pH 7.4) was added in a volume to obtain a

ratio of tissue/buffer approximately 1:4 w/v (i.e. 1g of liver to 4 ml of buffer B). The tissue was homogenized thoroughly using several short thrusts of the homogenizer's rotor. The homogenates were centrifuged at a speed of 15,000 g for 20 min at 4^{o}C. The supernatants were transferred to ultracentrifuge tubes, and the remaining pellet was re-homogenized with buffer B and centrifuged as described above. The supernatants from these preparations were transferred to ultracentrifuge tubes and centrifuged at 100,000g for 60 min at 4^{o}C. The pellets were washed with 0.1M Phosphate buffer (pH 7.4) and centrifuged at 100,000g for 60 min at 4^{o}C. The supernatants were discarded and the remaining pellets were reconstituted in buffer C (0.05M Tris, 1mM EDTA, pH 7.4, 20% Glycerol) to obtain a protein concentration 20 - 50 mg/ml. In order to uniformly dissolve the pellet, this preparation was briefly homogenized. Aliquots of the microsomal preparation were transferred to plastic vials (approx. 1 ml in each) and stored at -80^{o}C. Microsomal protein was determined in the micro-plate format using the Advanced Protein Assay Kit (Fluka) with bovine serum albumin as a standard.

3.2.5 Quantification of Cytochrome P450, Cytochrome b5, and Cytochrome P450 Reductase

Quantification of Cytochrome P450: Cytochrome P450 content in the microsomal preparation was determined according to the procedure described by Omura and Sato (1964a, b). Briefly, diluted microsome preparation was mixed with a few crystals of sodium dithionite in order to reduce cytochrome P450, which was used as a reference blank (baseline). The samples were then saturated with carbon monoxide for 30 sec, and the absorbance at 420 nm, 450 nm, and 490 nm was measured. The CYP450 content was determined by using the extinction coefficient (ΔE450-490) of 91 mM^{-1} cm^{-1}.

Quantification of cytochrome b5: Cytochrome b5 was determined from the difference in spectrum between NADH-reduced and air-saturated microsomes. The microsomal preparation was diluted in 0.1M phosphate buffer pH7.4 to gain the final protein concentration of 2mg/ml. The sample saturated with air bubbles was used as blank before the addition of NADH. NADH was added to the microsome suspension to obtain the final concentration of 0.2mM. Spectra at 424nm and 409nm were recorded. The

content of cytochrome b5 was determined using a molar extinction coefficient of 185 $mM^{-1}cm^{-1}$.

Assay for NADPH-cytochrome P450 reductase activity: Enzyme activity was monitored using rates of potassium ferricyanide reduction at 30°C in 0.3 M, pH 7.7 phosphate buffer (Schellenberg and Hellerman, 1958). The reaction mixture contained 0.5mM potassium ferricyanide and microsomal preparation with final protein concentration of approximately 50µg/ml. The reaction was initiated by the addition of 10mM NADPH to the final concentration of 0.1µM. The rate of the reduction was then determined by measurement of the reduced product of the reaction using extinction coefficient of 1.02 mM^{-1} cm^{-1} at 420 nm. The reaction without microsomal protein was used as the background.

3.2.6 Statistical analysis

Statistical analysis was carried out by ANOVA using the microcomputer package Number Cruncher Statistical System (HINTZE, 1995). The means were compared using Fisher's LSD test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

3.3 Method Validation

The above-described procedures were validated using recovery, purity, reproducibility, stability and linearity tests.

3.3.1 Cytochrome P450 Recovery

To identify the recovery of the isolation procedure, gopher liver tissue was processed on 4 different occasions. After the homogenate was centrifuged, the supernatant was saved and the pellet was re-homogenized, which was repeated three times. The respective preparations were centrifuged at 100,000g. The supernatants were discarded and the microsomal pellets were collected. The cytochrome P450 content in each extraction fraction of microsomal preparation was measured in the same way. The total content of cytochrome P450 in the sample of gopher liver was 21.85nmol/g liver tissue on

average. The cytochrome P450 content in the first extraction was 13.82nmol/g liver (63.2% of the total content), which was 6.47nmol/g liver (29.6% of the total content) in the second extraction, and1.56nmol/g liver (7.2% of the total content) in the third extraction. There was no detectable cytochrome P450 in the last extraction. The first extraction was combined with the second extraction to yield a good recovery (92.8% of the total content).

3.3.2 Cytochrome P450 Purity

In order to increase the enzyme purity, microsomal preparations were further washed with phosphate buffer (0.1M, pH 7.4). Washing improved microsome quality with clearly delimited absorbance at 450 nm and no detectable absorbance at 420 nm. When cytochrome P450 enzymes are denatured or inactivated, a shift occurs in the peak absorbance from 450 nm to 420 nm. Hence, the denatured or inactivated form of microsomal enzymes in this experiment was undetectable. Furthermore, this procedure will allow us to clearly distinguish the losses of activity associated with intrinsic variables from those caused by procedural variables.

3.3.3 Cytochrome P450 Stability

The stability of cytochrome P450 under different storage conditions was investigated. No significant decreases in microsomal levels or cytochrome P450 enzymes were observed by storage at -20°C or room temperature for 24h.

3.3.4 Linearity

In order to test the cytochrome P450 relationship with respect to its content and dilution, different dilution ratios of the extracted microsomal fractions were used in the measurement of the cytochrome P450 content. A plot was constructed where absorbance (A450-A490) was graphed versus dilution ratio. The trend line ($Y=1.1639X-0.0025$, $R^2=0.9981$) showed a high degree of correlation between the dilution ratio of microsomal preparation and the cytochrome P450 content.

3.3.5 Range

The protein standard curve was established using a 96 well microplate reader. The standard curve range was decided by a series dilution of BSA protein. The relationship became nonlinear when the concentration exceeded 1.2mg/ml. The range from 18.75---300ug gave a good linearity range (R^2=0.997). The microsomal preparation for assays was properly diluted to be within this linear range.

3.3.6 Reproducibility

The above described quantitative measurement procedure was repeated 5 times. The data obtained showed a high degree of reproducibility with a percent coefficient of variation (CV) of 1.33 for the measurement of cytochrome P450 content, and a %CV of 2.24 for microsomal protein measurements.

3.4 Results

Liver and liver microsomal protein concentration: The livers were successfully perfused (Figure 3.1). Table 3.1 presents the data on liver weight relative to body weight, as well as liver microsomal protein content. Table 3.1 indicates no significant differences in relative liver weight between male and female gophers, but in comparison to naïve, exposed gophers had significantly ($P<0.001$) larger livers relative to their body weight. Liver microsomal protein contents did not differ significantly either between males and females or naïve and exposed gophers. However, exposed gophers had higher liver microsomal protein contents per unit of body weight ($P<0.044$). The effect of status (i.e. toxin exposure) on females tended to be higher than that of males for liver (% BW); however, the interaction between gender and status was not statistically significant ($P = 0.09$). Furthermore, no significant interaction between gender and status was observed for microsomal protein concentration.

Liver microsomal cytochrome P450 enzyme system: Table 3.2 shows data for liver cytochrome P450 content. Significant differences were observed between exposed and naïve gophers in cytochrome P450 content when expressed per unit of microsomal

protein (P<0.037) and when expressed per unit of liver weight (P<0.012). Furthermore, when expressed normalized to body weight, the differences in cytochrome P450 content were considerably more pronounced (P<0.001). The differences between naïve and exposed gophers were significant for both males and females, but there were no significant differences between naïve males and naïve females or between exposed males and exposed females. There were no significant interactions between gender and status for liver cytochrome P450 content.

Table 3.3 shows data for liver microsomal cytochrome b5 content. No significant differences were observed between exposed and naïve gophers in cytochrome b5 content when expressed per unit of microsomal protein or when expressed per unit of liver weight. However, when expressed per unit of body weight, the differences between naïve and exposed gophers were significant for both males and females (P<0.026). There were no significant differences between naïve males and naïve females or between exposed males and exposed females. When considering the interaction between gender and status, the effect of status on females for the cytochrome b5 content based on protein content was significant higher than that on males (P<0.01). When expressed per unit of body weight, the effect of status on females tended to be higher than males (P=0.08).

Table 3.4 shows data for the liver microsomal cytochrome P450 reductase activity. There were no significant differences between exposed and naïve gophers in the activity of cytochrome P450 reductase activity when expressed per unit of microsomal protein or when expressed per unit of liver weight. However, the exposed gophers tended to have higher cytochrome P450 reductase activity when expressed as per unit of body weight (P<0.062), and the differences between naïve and were more pronounced for females (P<0.05). There were no significant differences between naïve males and naïve females or between exposed males and exposed females. When considering the interaction between gender and status, no statistical difference was observed for cytochrome P450 reductase activity.

Figure 3.2 illustrates the magnitude of the relative differences in various

components of the hepatic cytochrome P450 system between naïve and exposed gophers. When expressed on the basis of body weight, all important components of the hepatic microsomal detoxification system were considerably higher in gophers that were previously exposed to toxins used for their chemical control (Figure 3.2). Also noteworthy is that this apparent response was considerably stronger in females than in males.

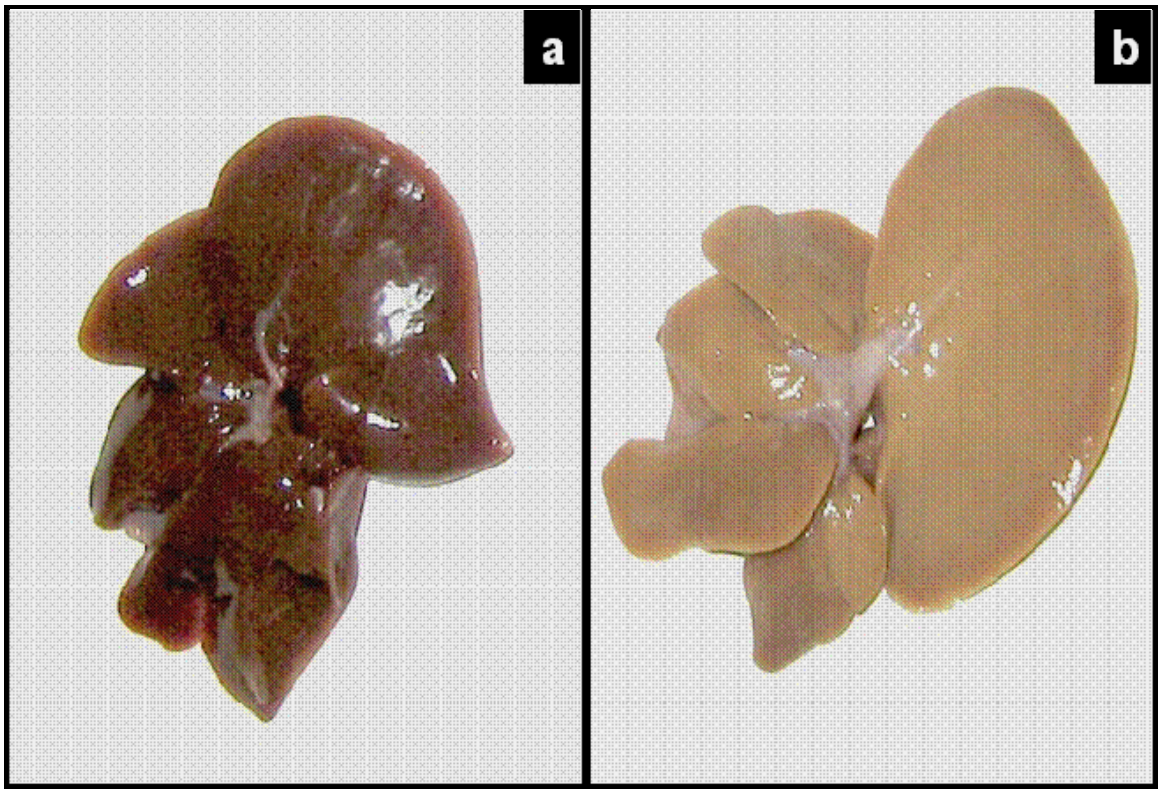

Figure 3.1. Fresh (a) and perfused (b) liver harvested from a gopher. Noteworthy is a pale appearance of the perfused specimen which is indicative that blood was successfully removed from the tissue.

Table 3.1. Relative liver weight indices and the content of microsomal protein in naïve gophers and those previously exposed to toxins used for their control (mean ± SE).

Subject of Study	**Body Weight** (g)	**Liver** (% BW)	**Microsomal Protein** (mg/g Liver)	**Microsomal Protein** (mg/100g BW)
Females Naïve	219.18 ± 15.62[a]	3.39 ± 0.23[a]	14.08 ± 2.03[a]	46.47 ± 5.88[a]
Females Exposed	318.02 ± 9.31[b]	4.20 ± 0.06[b]	15.06 ± 1.83[a]	62.78 ± 6.60[a]
Males Naïve	277.27 ± 15.51[c]	3.62 ± 0.08[a]	12.54 ± 2.14[a]	44.90 ± 7.31[a]
Males Exposed	313.03 ± 6.10[bc]	3.96 ± 0.07[b]	14.10 ± 1.28[a]	56.11 ± 5.69[a]
ANOVA (gender)	*P=0.044*	*P=0.939*	*P=0.51*	*P=0.53*
ANOVA (status)	*P<0.001*	*P<0.001*	*P=0.50*	*P<0.044*
ANOVA (Gender x status)	*P=0.018*	*P=0.09*	*P=0.88*	*P=0.69*

The values are means of six animals; SE=standard error. Means were compared using Fisher's LSD, $\alpha = 0.05$, means with the same letters are not significantly different.

Table 3.2. Liver cytochrome P450 content in naïve gophers and gophers previously exposed to toxins used for their control (mean ± SE).

Subject of Study	CYP450		
	(nmol/mg protein)	(nmol/g Liver)	(nmol/100 g BW)
Females Naïve	0.64 ± 0.05^{a}	8.69 ± 0.99^{a}	29.07 ± 3.24^{a}
Females Exposed	0.85 ± 0.05^{ab}	12.83 ± 1.75^{b}	53.54 ± 6.60^{b}
Males Naïve	0.81 ± 0.10^{ab}	9.48 ± 1.38^{a}	33.95 ± 4.52^{a}
Males Exposed	0.93 ± 0.07^{b}	12.79 ± 1.15^{ab}	50.62 ± 4.59^{b}
ANOVA (gender)	*P=0.102*	*P=0.776*	*P=0.843*
ANOVA (status)	*P<0.037*	*P<0.012*	*P<0.001*
ANOVA (gender x status)	*P=0.49*	*P=0.76*	*P=0.43*

The values are means of six animals; SE=standard error. Means were compared using Fisher's LSD, $\alpha = 0.05$, means with the same letters are not significantly different.

Table 3.3. Liver microsomal cytochrome *b5* content in naïve gophers and gophers previously exposed to toxins used for their control (mean ± SE).

Subject of Study	Cytochrome b5		
	(nmol/mg protein)	(nmol/g Liver)	(nmol/100 g BW)
Females Naïve	0.51 ± 0.04^{a}	7.26 ± 1.31^{a}	24.32 ± 4.14^{a}
Females Exposed	0.65 ± 0.04^{b}	9.64 ± 1.00^{a}	40.31 ± 3.86^{b}
Males Naïve	0.66 ± 0.05^{b}	7.94 ± 1.12^{a}	28.56 ± 3.86^{a}
Males Exposed	0.56 ± 0.04^{ab}	7.73 ± 0.71^{a}	30.67 ± 3.04^{ab}
ANOVA (gender)	*P=0.525*	*P=0.567*	*P=0.48*
ANOVA (status)	*P=0.648*	*P=0.317*	*P<0.026*
ANOVA (gender x status)	*P<0.01*	*P=0.24*	*P=0.08*

The values are means of six animals; SE=standard error. Means were compared using Fisher's LSD, $\alpha = 0.05$, means with the same letters are not significantly different.

Table 3.4. Liver microsomal cytochrome P450 reductase activity in naïve gophers and gophers previously exposed to toxins used for their control (mean ± SE).

Subject of Study	CYP450 reductase		
	(µmol/min/mg protein)†	(µmol/min/g Liver)	(µmol/min/100 g BW)
Females Naïve	0.22 ± 0.02[a]	3.02 ± 0.47[a]	10.10 ± 1.45[a]
Females Exposed	0.25 ± 0.02[a]	3.77 ± 0.46[a]	15.75 ± 1.69[b]
Males Naïve	0.27 ± 0.03[a]	3.39 ± 0.59[a]	12.18 ± 2.05[ab]
Males Exposed	0.24 ± 0.03[a]	3.32 ± 0.35[a]	13.16 ± 1.45[ab]
ANOVA (gender)	*P=0.365*	*P=0.934*	*P=0.884*
ANOVA (status)	*P=0.961*	*P=0.483*	*P<0.062*
ANOVA (gender x status)	*P=0.17*	*P=0.40*	*P=0.18*

The values are means of six animals, SE=standard error. Means were compared using Fisher's LSD, α = 0.05, means with the same letters are not significantly different, †reduced ferricyanide.

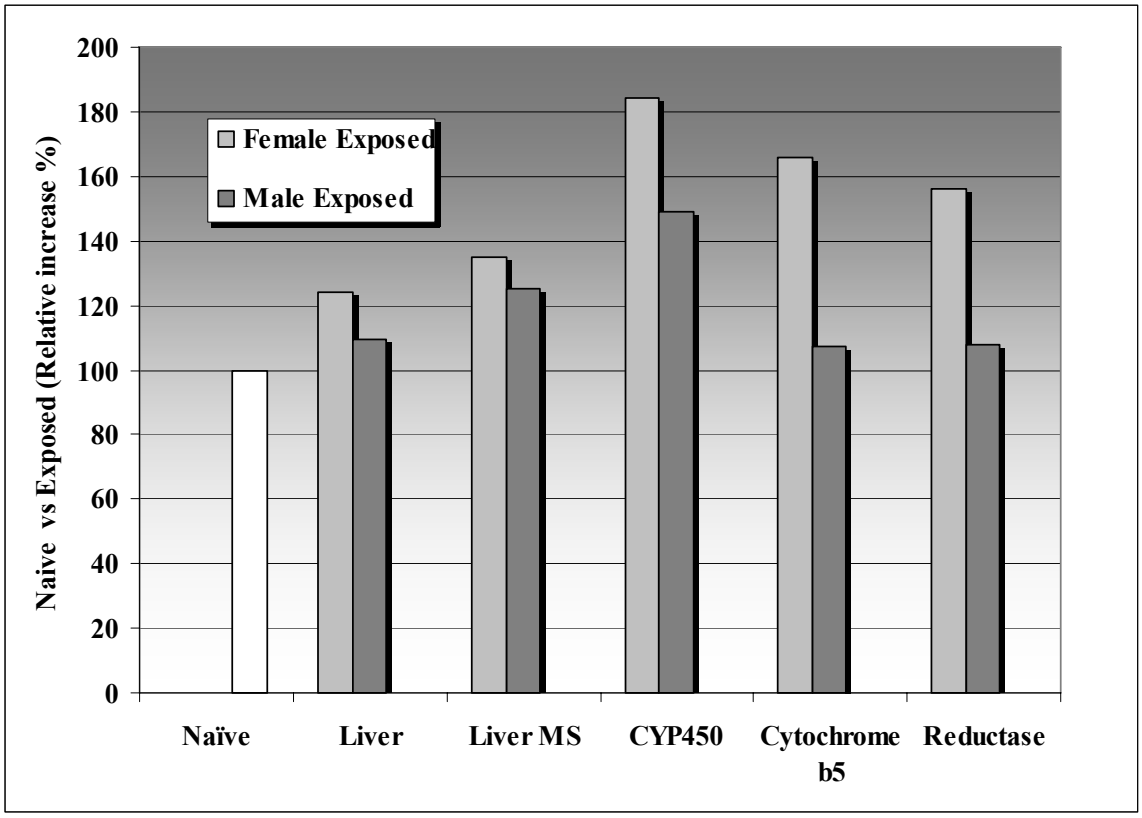

Figure 3.2. Relative differences in various components of the hepatic cytochrome P450 system between naïve and exposed gophers. White bar on the graph indicates basic activity of naïve gophers (100%), and this is compared to relative increases for exposed males (dark grey bars) and females (grey bars).

3.5 Discussion

The gopher problem mainly affects forage or livestock production. Since the early days of intensive farming on the prairies, farmers committed an extensive amount of resources to control gopher populations. Chemical toxicants, such as strychnine and zinc phosphide, can cause an immediate mortality of more than 70% (Matschke et al., 1983). Despite these measures, a recent survey (2001 Saskatchewan "Gopher" Survey) suggests that the gopher population continues to increase. In addition, during the trapping, we noted severe gopher infestation, with no apparent difference in gopher densities, on sites with or without toxicological control measures. This observation indicates that the current chemical methods of gopher control may be not effective. Although limited in scope, our data is generally in agreement with the information provided from the Saskatchewan "Gopher" survey. Possibly gophers previously exposed to toxic baits in many areas of the province may have developed resistance to such toxins. This is a significant impediment in the attempts to control gopher numbers.

Scientific data is not available to explain the decreasing efficiency of poison control methods used for gophers. However, precedence exists for pests to develop resistance to toxins if they are exposed to sub-lethal doses of poison and to pass this resistance on to their offspring (NRC, 2000). One potential mechanism of resistance is modification of their metabolism. For instance, the activities of the cytochrome P450 enzyme system may increase when the host is exposed to sub-lethal levels of toxins (Klassen, 2001). These enzymes are largely responsible for adaptation of pests to toxins. Over time this adaptation may render even potent toxicant doses as ineffective (NRC, 2000). The enhanced toxin resistance may be inherited from the parent population who were previously exposed to the poison (Agrawal and Shapiro, 1996).

The liver contains the bulk of the detoxification potential available for the host. Notably, the exposed gophers had larger livers relative to their body weight. Due to the fact that there were no significant differences in microsomal protein per unit of liver weight, it can be inferred that this additional accrual of liver mass by the host was an effective metabolic investment directed towards increased overall functional capacity of

the organ to allow for more efficient detoxification.

The cytochrome P450 enzyme system is comprised of a family of catalytic enzymes with very low substrate specificity designated to detoxify a wide range of xenobiotics (Walker, 1983). The hepatic cytochrome P450 system is the most important component of toxin metabolism responsible for biotransformation of compounds entering the body via gastrointestinal tract (Klassen, 2001). The efficiency and detoxifying potential of this system plays a crucial role in determining the magnitude and duration of toxic effects (Klassen, 2001). Liver microsomal cytochrome P450 system is comprised of three, functionally different, enzyme entities that include the cytochrome P450 family of catalytic enzymes, cytochrome b5, and cytochrome P450 reductase (Klassen, 2001).

Our experimental findings that the content of hepatic cytochrome P450 enzymes is considerably elevated in gophers that were previously exposed to toxins is of practical significance. In particular, the magnitude of the differences between exposed and naïve gophers in cytochrome P450 family of catalytic enzymes require thorough deliberation. This may help to explain the decreased efficiency of current chemical methods used to control the population of gophers, which will be thoroughly explained in chapter 7.

Both cytochrome b5 (a donor of second electron for the oxidative reactions) and cytochrome P450 reductase (enzyme reducing the NADP cofactor that is vital for the catalytic reactions mediated by cytochrome P450 family of enzymes) are constitutive proteins. Therefore, it was not surprising that these proteins showed no significant increases when expressed per unit of microsomal protein or when expressed per unit of liver weight. On the other hand, hepatic cytochrome P450 family of catalytic enzymes are inducible when animals are exposed to toxins, and this explains why cytochrome P450 content was higher in gophers previously exposed to toxins in comparison to the naïve gophers. However, CYP450 proteins only constitute a limited percentage of total microsomal protein content. Another main microsomal enzyme system involved in pesticides metabolism is flavin-containing monooxygenase (FMO) system (Hodgson et al., 1995). This system is also inducible despite the fact that FMO enzymes are not

under the same regulatory control as cytochrome P450 enzymes (Parkinson, 1996). Thus, the difference of microsomal protein content between naïve gophers and exposed gophers may be the result from induction of both cytochrome P450 system and FMO system.

The capability of an animal to survive adverse effects of ingested toxins must be considered from the perspective of the potency of the detoxification system. A useful index of the potency of the detoxification system is the amount of enzyme involved in the detoxification per unit of body weight. Notably, all important components of the hepatic microsomal detoxification system were considerably higher in gophers that were previously exposed to toxins used for their chemical control. In comparison to naïve gophers, the level of cytochrome P450 enzymes in exposed gophers increased by 84.2% and 49.1% for females and males, respectively. Hence, it is evident that gophers have tremendous capacity to increase their detoxifying power when exposed to toxins. Also noteworthy is the considerably higher detoxification potential in female gophers in comparison to male gophers. From an evolutionary point of view, this may be a very efficient adaptive strategy of the species to guarantee survival. In order to ensure reproductive success in the face of toxicity, the survival of female gophers may be more important than the survival of male gophers.

The recent 2001 Saskatchewan "Gopher" Survey indicates that gophers thrive despite attempts to control them. Resistance of gophers to toxins may be a significant impediment in the attempts to control their population. Our findings indicate that gophers may readily develop resistance to toxins by enhancing the functional capacity of enzymes responsible for detoxification. This may explain the tremendous ability of this pest to thrive despite constant attempts to control this pest for several decades. However, in this context it is important to understand that in such situations it may be necessary to constantly adjust the dose of the toxin to obtain the desirable lethal effect.

In conclusion, our studies suggest that gophers may improve their detoxification capacity by enhancing expression levels of cytochrome P450 enzyme system components when they become exposed to toxins. This adaptive response may be

exploited to design and develop new, more effective, species-specific and ethically acceptable technologies to control gopher populations. However, whether this is the only reason for the decreasing efficiency of current rodenticides is still not clear. Further investigation will be required to identify all underlying causes for the loss of rodenticide efficiency.

4.0 IN VITRO METABOLISM OF SELECTED SUBSTRATES BY MICROSOMAL CYTOCHROME P450 SYSTEM IN GOPHERS

4.1 Introduction

Recently, the efficiency of pesticides commonly used to control gopher population compounds has been questioned. Also, the problems associated with animal welfare have become a concern. Further, because of lack of specificity, the currently used pesticides present a high risk of primary and secondary toxicity to non-target animals. Thus, there is an urgent need for the development of a more efficient, species specific and socially acceptable means of control in order to provide long term solutions to these problems.

The most effective delivery method for pesticides is through ingestion. Liver is a primary organ involved in detoxification of orally administered toxins. The magnitude and duration of toxic effects greatly depends on the catalytic activities of hepatic biotransformation enzymes, and the hepatic cytochrome P450 enzymes involved in xenobiotic biotransformation is of primary importance.

The activity of the cytochrome P450 system in gophers has not yet been studied. A better understanding of detoxification mechanisms in gophers may provide valuable information that may help in the design of more effective chemical means of gopher population management. Accordingly, our primary objectives were to evaluate the activity of major detoxification pathways of hepatic cytochrome P450 system. Several compounds discussed below were used as general probes to evaluate the kinetics of major biotransformation reactions *in vitro*. Coumarin and aniline were selected as the probes to examine aromatic hydroxylation. In order to evaluate the activity of enzymes involved in N-dealkylation, N,N-dimethylaniline and N-methylaniline were used. 7-methoxycoumarin was selected as a probe to evaluate O-demethylation pathways.

Coumarin occurs naturally in various plants. The food and tobacco industries have used coumarin as a flavoring for a century. The toxicity of coumarin has been comprehensively investigated. The liver is the major target organ for coumarin-induced toxicity, which often causes hepatocyte necrosis (Lake and Grasso, 1996). Species differences exist in the capacity to form the 7-hydroxylated metabolite of coumarin (7-OH-coumarin) (Cohen, 1979; Fentem and Fry 1992; Fentem and Fry 1993). Rodents have been identified to have greater susceptibility to coumarin toxicity than other animals including humans since they produce minor quantities of 7-OH-coumarin (Born et al., 2003). The LD_{50} for rat was reported to be 293 mg/kg (Lewis, 1996). This means that rodents are more susceptible to coumarin or coumarin derivatives.

Aniline is a man-made chemical, which can be used in manufacturing of herbicides and fungicides (Canadian Environmental Protection Act, 1994). Aniline may induce spleen hemangiosarcoma, as well as DNA damage *in vivo* in rat livers and kidneys. Aniline has an oral LD_{50} of 250 mg/kg for rat. Aniline is largely metabolized to 4-aminophenol (Parke, 1968). The measurement of 4-aminophenol formation has been traditionally used to quantify the 4-hydroxylation of aniline (Stevens et al., 1980).

The initial oxidative metabolism of many secondary and tertiary N-alkylamines, including N-methylaniline (NMA) and N,N-dimethylaniline (DMA), by liver microsomes are catalyzed by either cytochrome P450 system or the mixed-function amine oxidase (Pandey et al., 1989). The final N-demethylation product of DMA is NMA, and for NMA, aniline is the end product (Sherratt and Damani, 1989). There is limited evidence in experimental animals on the carcinogenicity of N,N-dimethylaniline (U.S. Environmental Protection Agency, 1999).

7-Methoxycoumarin (7-MC) is another substrate of the cytochrome P450 enzyme system investigated in this study. This compound will go through dealkylation and primarily form 7-hydroxycoumarin as the end product (Reen et al., 1991).

Chemical structures of the above discussed substrates are presented in Figure 4.1. In the present study, these substrates were tested using hepatic microsomes from both sexes of gophers from naïve and previously exposed to toxin in field sample groups.

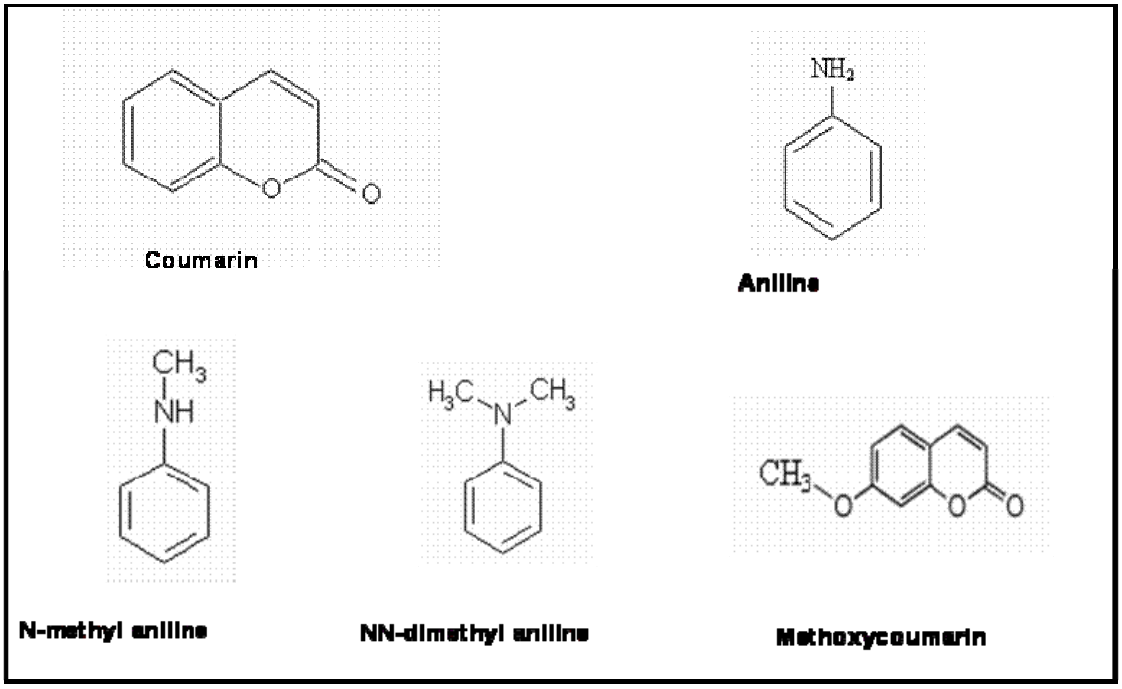

Figure 4.1. Chemical structures of substrates selected for the study of major detoxification reactions including aromatic hydroxylation using coumarin and aniline (Sigma-Aldrich, Inc., U.S.A.) as probe substrates, N-demethylation using N-methylaniline and N,N-dimethylaniline (Sigma-Aldrich, Inc., U.S.A.) as probe substrates, and O-demethylation using 7-methoxycoumarin (Sigma-Aldrich, Inc., U.S.A.) as probe substrate.

4.1.1 Rationale

Estimation of the Michaelis-Menten parameters from liver microsomes study is practically important for our purpose, which can be illustrated in the following table:

	Toxin: High inherent toxicity Metabolites: Low toxicity		Toxin: Low inherent toxicity Metabolites: High toxicity	
	Level	Outcome (Toxicity)	Level	Outcome (Toxicity)
V_{max}(nmol/mg)	High	Low	High	**High**
	Low	**High**	Low	Low
K_m(μM)	High	**High**	High	Low
	Low	Low	Low	**High**

For a toxin with high inherent toxicity but relatively low toxicity for its metabolites, poor metabolic efficiency may indicate a higher potential for toxicity. Hence, cytochrome P450 pathways involved in its biotransformation *in vitro* that demonstrate both low V_{max} and high K_m values may suggest a strong potential for significant *in vivo* toxicity. Low V_{max} values indicate relatively low rates of detoxification. Such toxins will accumulate in the body and cause adverse effects, and eventually death. High K_m values indicate a poor affinity of the toxin for that particular cytochrome P450 pathway and high toxin concentrations are needed to reach detoxification rates at 50% of maximal velocity. This allows the existing toxin to distribute at high levels throughout the body to cause adverse effects and possibly death.

For toxins with low inherent toxicity, but with highly toxic metabolites, cytochrome P450 pathways involved in its biotransformation *in vitro* that demonstrate both high V_{max} values and low K_m values favor the potential for high *in vivo* toxicity. In this situation high V_{max} values indicate a rapid turnover of toxin to its toxic metabolites. Depending upon the rate of metabolite elimination, toxic metabolites may accumulate in

the body and contribute to high toxicity. Furthermore, low K_m values indicate that even though a subtle amount of toxin is introduced into the body, it can be rapidly transformed into toxic metabolites. The accumulation of such toxic metabolites may pose a serious health risk.

4.2 Materials and Methods

4.4.1 General incubation conditions

Microsomal samples were prepared as described in chapter 3. For the assays, the preserved stock preparations of microsomes were reconstituted in 0.1M Phosphate buffer (pH 7.4) at the optimal concentration. The optimal concentrations of microsomal proteins for the assays were determined from preliminary experiments (for details see later). Determination of metabolite formation rates provided estimates for the Michaelis-Menten parameters, V_{max} and K_m, for each of the evaluated substrates. Reactions were carried out in 1.5 mL Eppendorf vials. The amount of organic solvents in the final reaction mixtures did not exceed 5% (v/v).

The final volume of the reaction mixture was 100 µl in phosphate buffer (0.1 M, pH 7.4). The reaction mixture was comprised of 95 µl of microsomal suspensions and 5 µl substrate dissolved in methanol, to which 5 µl NADPH (10mM) dissolved in phosphate buffer (final concentrations: 0.5 µM for assays) or 5 µl of buffer (for blank) were added. The reaction vials were then incubated in a shaking water bath at 37°C open to the ambient air. The optimal concentrations of microsomal protein, substrates, and the length of the incubation period to give linear metabolite formation conditions were established for all substrates during method validation. Eight concentration levels were used for each substrate selected, with the highest concentration being added to the blank (no NADPH). For the selected substrates, then, these optimization experiments ensured that the rates of product formation were linear with time and protein concentration for all enzymatic activities under study. Reactions were terminated by adding 100 µl of acetonitrile. This termination method effectively stopped the reaction. No changes in concentration of metabolites were observed in the vials even after 24

hours at room temperature. For this procedure, enzymatic reactions were quantified using authentic metabolite standards. The standards used in our study were prepared using the same conditions as the reaction mixture without substrates as described above.

4.4.2 HPLC analysis

After the reaction was terminated, reaction mixtures were centrifuged for 30 min at 14,000 rpm (Microfuge 18, centrifuge, Beckman, Coulter, Germany). The supernatants were then filtered through a 0.45 μm syringe filter and 10 μl was injected onto the HPLC. Selected substrates and their metabolites were separated using a reversed-phase column (silica-based C_{18} (2), LUNA, 150 x 4.60mm, 3μ, Phenomenex, Torrance, CA), an HPLC system with autoinjector (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) and fluorescence detector (RF-551, Spectrofluorometric detector, SHIMADZU, Columbia, MD, U.S.A.). The methods were validated as described in method validation section.

4.4.3 Specific experimental parameters for each substrate

4.4.3.1 Coumarin aromatic hydroxylation

Coumarin concentrations ranged from 1.56 μg/ml to 100 μg/ml. Microsomal protein concentration was approximately 500 μg/ml, and the reaction mixture was incubated for 5 min. The metabolite standard for coumarin metabolism was 7-OH-coumarin (Fluka, Switzerland). The metabolite and coumarin were separated using 40% methanol (Methanol:ddH_2O) as Mobile Phase (MP) at 0.8ml/min flow rate. Detection parameters were: Excitation wavelength (E_x) 325 nm and Emission wavelength (E_m) 445 nm.

4.4.3.2 Aniline aromatic hydroxylation

Aniline concentrations ranged from 1.95 μg/ml to 250 μg/ml. The microsomal protein concentration was approximately 1 mg/ml, and the incubation time of the reaction mixture was 30 min. Metabolite standard for aniline metabolism was 4-

aminophenol. The metabolites and aniline were separated by using 50% methanol (Methanol:ddH_2O) as MP at 0.8ml/min flow rate. Detection parameters were: E_x 299 nm and E_m 367 nm.

4.4.3.3 N-methylaniline and N,N-dimethylaniline N-demethylation

N-methylaniline concentration ranged from 3.91 μg/ml to 500 μg/ml. The microsomal protein concentration was approximately 0.8 mg/ml. The N,N-dimethylaniline concentration ranged from 0.98 μg/ml to 125 μg/ml. Incubation times were 5 min for both substrates. Metabolites standards for N-methylaniline and N,N-dimethylaniline metabolism were aniline and N-methylaniline, respectively. The metabolites and parent compounds were separated by using 70% methanol (Methanol:ddH_2O) as MP at 0.8ml/min flow rate. Detection parameters were: E_x 285 nm and E_m 345 nm.

4.4.3.4 7-Methoxycoumarin O-demethylation

7-Methoxycoumarin concentration ranged from 0.20 μg/ml to 25 μg/ml. The microsomal protein concentration was approximately 100 μg/ml, and the incubation time was 5 min. The metabolite standard was 7-OH-coumarin. The metabolite and 7-methoxycoumarin were separated by using 50% methanol acidified with citric acid (pH 4.07) as MP at 0.8ml/min flow rate. Detection parameters were: E_x 299 nm and E_m 367 nm.

4.4.4 Enzyme Kinetics

Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were used to define the enzymatic activity. K_m and V_{max} values were determined by use of nonlinear regression analysis (rate of metabolite formation against substrate concentration). GraphPad Prism 3.03 software for Windows (GraphPad Software, San Diego, CA) was used for data analysis. Both one binding site model and two binding site model were evaluated. The 95% confidence interval was evaluated to choose between one-enzyme site and two-enzyme site model. K_m and V_{max} values were determined using one site

binding model (Figure 4.2, Figure 4.3).

4.4.5 Statistical Analyses

All data are presented as mean ± standard error (SE). Statistical analyses were carried out using ANOVA from the microcomputer package Number Cruncher Statistical System (HINTZE, 1995). The means were compared using Fisher's LSD test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

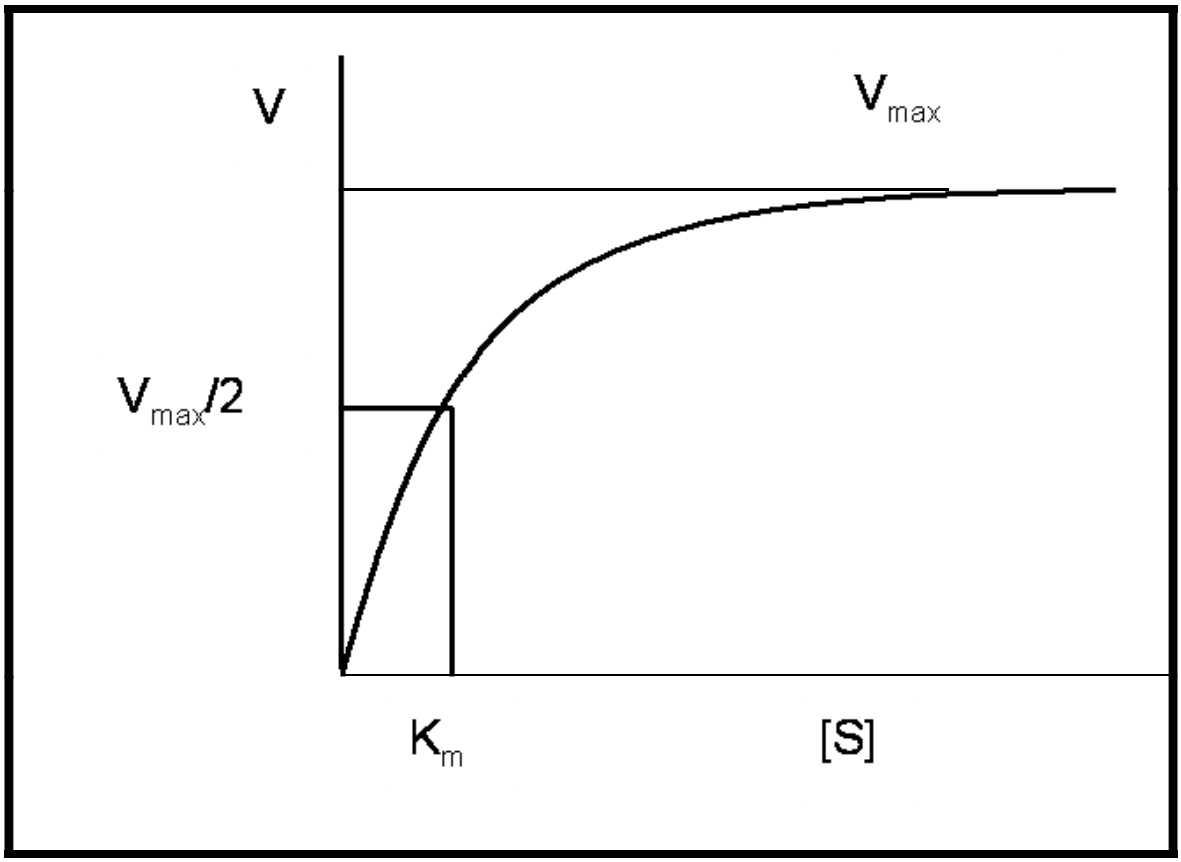

Figure 4.2. Hepatic metabolism follows: increase in reaction velocity (V) with increase in substrate concentration ([S]). As the substrate concentration increases, saturation of substrate binding to the enzyme active site eventually occurs and a maximal reaction velocity (V_{max}) is reached. The substrate concentration at a reaction velocity which is half V_{max} is called the K_m and is a measure of the affinity of the enzyme for the substrate. The reaction velocity (V) at any particular substrate concentration ([S]) is given by one site binding model in our study:

$$V = \frac{V_{max} \times [S]}{K_m + [S]}$$

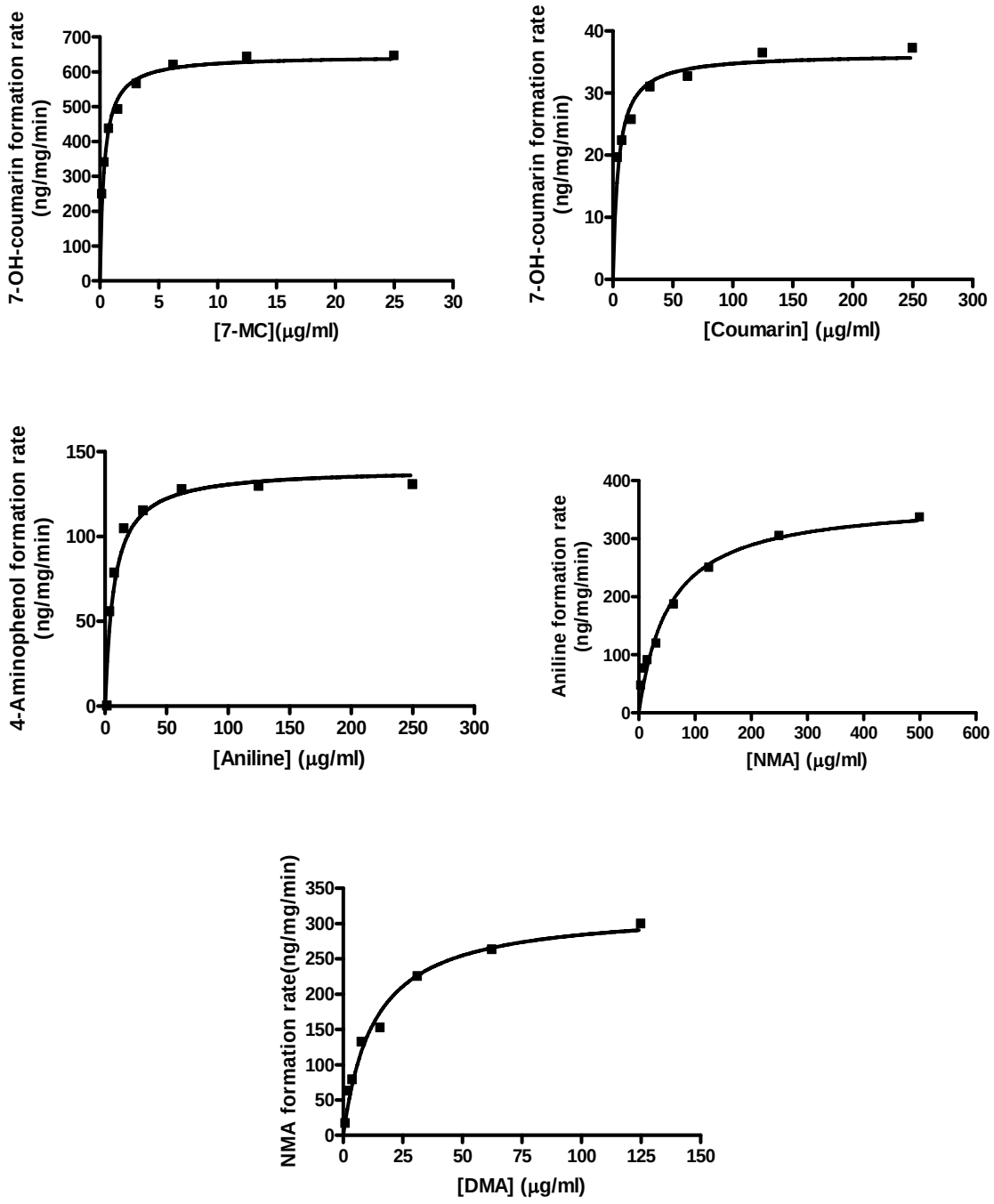

Figure 4.3. Representative plots of enzyme kinetic data obtained from *in vitro* metabolism of various compounds by CYP450 system. The individual data points were fitted in the Michaelis-Menten kinetics model using one binding site model (GraphPad Software).

4.5 Method Validation

For the selected substrates, optimal reaction and HPLC analysis conditions were determined through method validation procedures.

4.5.1 Coumarin Metabolism

4.5.1.1 Limit of Detection and Quantification

7-OH-coumarin of known concentration was diluted until the responses were 3-5 times that of baseline noise. The limit of detection for 7-OH-coumarin was 3.125 ng/ml. Furthermore, limit of quantification was determined by continued dilution of 7-OH-coumarin of known concentration until precision and accuracy no longer met appropriate standards (6.25—100ng/ml). The limit of quantification was 6.25 ng/ml.

4.5.1.2 Range and Linearity

A serial dilution of stock solution (100ng/ml) was used to establish the standard curve for 7-OH coumarin. Each calibration standard was analyzed in triplicate. The range from 6.25—100ng/ml gave a linear response (R^2 = 0.9998) with the response becoming nonlinear when the concentration reached 1μg/ml. As such, this range should cover all assay of coumarin metabolism.

4.5.1.3 Accuracy

7-OH-coumarin of known concentration was analyzed and a comparison of the measured value to the true value was performed to determine the accuracy of this procedure. The accuracy based on six replicates ranged from 97.2% to 102.2%.

4.5.1.4 Reagent Quality

Coumarin from different sources was tested on the HPLC to evaluate the quality of the reagent. It contained only trace of 7-OH-coumarin at very high concentration. There was no detectable amount of 7-OH-coumarin in the concentration used in the

present study. Thus, this amount will not affect the result of our kinetic study and the coumarin used in our experiment was of good quality.

4.5.1.5 Specificity of Column

Different columns were used to separate the potential metabolites and parent compounds. PRP-1 and silica-based C_{18} can be used under same conditions (40% methanol) to separate coumarin and 7-OH-coumarin, but the former one required 38 minutes for the elution of coumarin, whereas the later one required 8.8 minutes while maintaining a very good resolution. Silica-based C_{18} column was therefore chosen for its suitability for the separation of coumarin and 7-OH-coumarin.

4.5.1.6 Chromatographic Conditions

Different ratios of methanol and water (40%, 60%, 80% methanol) were used to determine the best mobile phase for the analysis. The mobile phases were degassed and delivered at a flow rate of 0.8 ml min^{-1}. Injection volume was 10µl for all samples and standards. 40% methanol gave better resolution than 60% and 80% methanol and consumed less solvent. Therefore, 40% methanol based MP was chosen optimum for the separation of coumarin and 7-OH-coumarin.

4.5.1.7 Linearity of Assay

Linear conditions for the formation of hydroxylated metabolite (7-OH-coumarin) were evaluated regarding protein content and incubation time for gopher hepatic microsomes.

Stock preparations of microsomes from gopher liver (containing 12 mg/ml of protein) were diluted 8, 16, 32, 64, and 128 times to provide different cytochrome P450 concentrations in 100µl total volume. Coumarin (5µl, 5mg/ml) was added to the reaction system (final volume 100µl) as a substrate. After 10 minutes pre-incubation in a shaking water bath at a constant temperature of 37°C, NADPH (5µl, 10mmol/l) was added to initiate the reaction. The reaction mixture was incubated in a shaking water

bath for 5 minutes at 37°C. Reactions were terminated by the addition of 100 µl acetonitrile. The samples were then vortex-mixed and centrifuged at 14,000rpm for 10 minutes to precipitate the protein. The supernatant was filtered and 10µl was injected to HPLC to separate the main metabolites (7-OH-coumarin) and the parent compound (coumarin). A linear relationship was obtained from the plot of product concentration against cytochrome P450 concentrations which indicated that the linearity was good under this condition ($R^2 = 0.9993$).

To test the relationship between time and enzymatic activity, the reaction mixture (0.1 ml total volume) including 0.5mg/ml microsomal protein, coumarin (5 µl, 5mg/ml), and NADPH (5µl, 10mM) was incubated in a shaking water bath kept at 37°C for 3, 6, 9, 12, 15, and 30 min. Triplicates were used for every point, and the reaction was ended by addition of acetonitrile. As the main metabolite, 7-OH-coumarin was determined by HPLC. A linear relationship was obtained when 7-OH-coumarin concentrations were plotted against time. The reaction remained linear up to 12 minutes ($R^2 = 0.9965$). Reactions became nonlinear at 15 minutes ($R^2=0.9883$), which may have been caused by the shortage of substrate since the cofactor (NADPH) was still detectable until 20 minutes.

The amount of metabolite (7-OH-coumarin) formed was linear with respect to the incubation time and amount of microsomal protein. The optimum conditions for the kinetics study were 0.5mg/ml protein concentration of microsomes at 37°C for 5 minutes incubation.

4.5.1.8 Reproducibility

In order to evaluate the reproducibility of the assay, same procedures were repeated under the optimum condition 4 times with a CV value of 2.37 %. This indicated a good reproducibility of the analytical procedure used in the present study, which was within the limits of 15% commonly considered as acceptable in experimental procedures (Guidance for Industry: Bioanalytical Method Validation, U.S. Food and Drug Administration).

4.5.2 Aniline, N-methylaniline, and N,N-dimethylaniline Metabolism

A desirable resolution was obtained using 70% methanol. The retention time (RT) for aniline was 2.15min, for N-methylaniline was 2.740min, and for N,N-dimethylaniline was 5.12min. For aniline metabolism, 4-aminophenol standard was separated from aniline using 50% methanol, with the RT for aniline 2.96min and 1.99 for 4-aminophenol.

The standard curve coefficients of determination for aniline, N-methylaniline, and N,N-dimethylaniline were routinely greater than $R^2 = 0.999$ under this condition. The standard curve was linear between 0.20μg/ml and 12.5μg/ml for aniline and N-methylaniline; whereas, for N,N-dimethylaniline the range was between 0.20μg/ml and 25μg/ml. Standard curves became nonlinear when the concentration was increased to 50μg/ml.

The accuracy for aniline was 99.8%, for N-methylaniline 101.7%, and for N,N-dimethylaniline 107.1%. The data obtained showed a high degree of reproducibility with a CV of 10.1% for aniline, 2.20% for N-methylaniline and 4.43% for N,N-dimethylaniline.

Linear conditions for the formation of metabolite (N-methylaniline for N,N-dimethylaniline; aniline for N-methylaniline; and 4-aminophenol for aniline) were evaluated regarding protein content and incubation time for gopher hepatic microsomes. A straight line graph was given when the products concentrations were plotted against microsomal protein concentrations (range from 0.4mg/ml to 1.4mg/ml), which indicated that the linearity was good under this condition (R^2=0.994 for N,N-dimethylaniline, R^2=0.9984 for aniline, R^2=0.996 for N-methylaniline).

The reaction remained linear up to 15 minutes for N-methylaniline and N,N-dimethylaniline, and 30 minutes for aniline. The amount of metabolites formed was linear with respect to the incubation time and amount of microsomal protein. The optimum conditions for the kinetics study were: 0.5-1mg/ml protein concentration of microsomes at 37°C for 5 minutes incubation, for N-methylaniline and N,N-

dimethylaniline, and 30 minutes for aniline.

4.5.3 7-Methoxycoumarin Metabolism

7-Methoxycouamrin and its metabolite 7-OH-coumarin were separated using acidified 50% methanol (pH 4.07) with RT 3.99 min for 7-OH-coumarin and 8.416 min for 7-Methoxycouamrin. The coefficient of determination value for the standard curve for 7-OH-coumarin was 0.9998. Linear metabolite formation kinetics was assessed as a function of microsomal protein concentration and time. A linear relationship was observed (R^2 = 0.9986) when the product concentrations were plotted against microsomal protein concentrations (range from 50 μg/ml to 400 μg/ml). The reaction remained linear up to 7 minutes (R^2 = 0.9973). Same procedure was done under the optimum condition 6 times and the CV% is 1.66 indicating good reproducibility

4.6 Results

4.6.1 Aromatic Hydroxylation

4.6.1.1 Coumarin Metabolism

The biotransformation of coumarin was not affected by either toxin exposure status or gender. However, naïve gophers tended to have higher K_m values than exposed gophers for both females and males. Naïve gophers had higher V_{max} values than exposed ones when normalized to cytochrome P450 content for both females and males. Although not statistically significant, males tended to have considerably higher specific activities than females. However, when extrapolating the data to the whole body activity exposed gophers had higher activity than naïve gophers for both genders (Table 4.1).

4.6.1.2 Aniline Metabolism

There were no significant differences between both toxin exposure status and gender for aniline metabolism. However, female naïve gophers tended to have higher K_m values than exposed, whereas male naïve gophers had slightly lower K_m values than exposed gophers. When comparing V_{max} values they followed the same pattern as

coumarin except when expressed based on whole body weight where female naïve gophers had slightly higher values than female exposed (Table 4.2).

When comparing coumarin and aniline metabolism, the K_m values of aniline was 2.1-fold higher than the K_m values of coumarin. Furthermore, specific activity was 3.5-fold higher even though variations existed between different groups for both substrates (Figure 4.4). No significant interactions between gender and status were observed for both K_m values and V_{max} values regarding aromatic hydroxylation.

4.6.2 N-demethylation

4.6.2.1 N-methylaniline Metabolism

The K_m values of naïve gophers are higher than exposed gophers for both sexes though the difference was not statistically significant. There was a significant difference when comparing K_m value within genders regardless toxin exposure status ($P<0.03$). The V_{max} values when normalized to cytochrome P450 content for naïve gophers were higher than that of exposed gophers. There was no statistical difference between naïve and exposed females, while there is statistical difference between naïve and exposed males for the V_{max} values based on the cytochrome P450 content. However, when calculated based on whole body activity, there were no significant differences for either sexes of naïve and exposed gophers. The effects of exposure status in males tended to be higher than that in female for both specific activity ($P=0.07$) and whole body activity ($P=0.06$) (Table 4.3).

4.6.2.2 N,N-dimethylaniline Metabolism

Unlike N-methylaniline, the K_m values for naïve gophers were lower than the exposed gophers with respect to both sexes and the difference between naïve and exposed females was statistically significant (Table 4.4). When only considering exposure status statistically significant differences in the K_m values existed between naïve and exposed gophers ($P<0.04$). There were no statistically significant differences for V_{max} values based on cytochrome P450 content between exposed and naïve female

gophers. However, the difference between naïve and exposed male gophers was statistically significant. In addition, when comparing calculated V_{max} values on a whole body basis, naïve and exposed gophers of both genders were not found to be statistically significant. Furthermore, a definite difference was observed where naïve gophers had higher K_m values than exposed gophers when calculated on the cytochrome P450 content (P=0.07). The effects of exposure status on females tended to be higher than that on male for K_m values (P=0.07). The effect of exposure status on males was significantly higher than females for specific activity (P=0.03). However, no statistically significant difference existed between the effect of status on genders for the whole body activity (P=0.05).

The K_m value of N-methylaniline was 2.6 times higher than N,N-dimethylaniline. However, the V_{max} values for N,N-dimethylaniline was 1.4-fold higher than N-methylaniline.

4.6.3 O-demethylation

4.6.3.1 7-methoxycoumarin Metabolism

Table 4.5 indicates no statistically significant differences for K_m values for all four groups. The V_{max} values based on cytochrome P450 content for naïve gophers were higher than exposed gophers with respect to both sexes, although the difference was not statistically significant. Nevertheless, when calculated on the basis of whole body activity exposed females had higher V_{max} values than naïve females and exposed males had slightly lower V_{max} values than naïve males. When considering males and females as the same groups, there was no difference between naïve gophers and exposed ones (P=1.00). No significant effects of status on gender were observed for both K_m and V_{max} (Table 4.5).

4.6.4 Comparison of Differences in Metabolism of Various Substrates

N-methylaniline metabolism had the highest K_m value in both exposed and naïve gophers, which significantly differed from the rest of the substrates. No significant

differences were observed between coumarin, aniline, and 7-methoxycoumarin with respect to K_m values. However, K_m values for N-demethylation were significantly higher than aromatic hydroxylation and O-demethylation. Cytochrome P450 enzymes tended to have the highest specific activity and whole body activity for 7-methoxycouamrin O-demethylation in both exposed and naïve gophers (Figure 4.5; Figure 4.6). Coumarin tended to have the lowest specific activity and whole body activity of cytochrome P450 enzymes in both exposed and naïve gophers. N-demethylation had significantly higher specific activity in both exposed and naïve gophers than aromatic hydroxylation, and O-demethylation had significantly higher specific activity and whole body activity than aromatic hydroxylation in all gophers (Table 4.6).

No significant differences existed between naïve and exposed gophers for intrinsic clearance for all the selected substrates (Table 4.7). However, 7-methoxycoumarin had the highest intrinsic clearance value when considering all the experimental gophers as one group as compared with the other compounds. N-Methylaniline had the lowest intrinsic clearance value as compared to the rest of the substrates (Table 4.7).

4.6.5 Differences within Population

No statistically significant differences were observed between gender and exposure status with respect to coumarin, aniline and 7-methoxycoumarin metabolism. However, upon closer analysis the data revealed an interesting trend. There was an identifiable subgroup of the population with distinctly different metabolic characteristics (Figure 4.7). Specifically, this subgroup was named as poor metabolizers (PM), which was defined as the individuals having less than 20% of the highest value of V_{max} based on 100g BW. The rest of the population was defined as strong metabolizers (SM).

Interestingly, 42% of the sample population could be described as PM group for coumarin, 25% for aniline and 29% for 7-methoxycoumarin. Enzymes responsible for aniline and 7-methoxycoumarin metabolism in PM had slightly lower affinity (higher K_m) than that in SM. However, the enzymes responsible for coumarin metabolism

differed in that PM had slightly lower affinity than the enzymes in SM. From the comparative data of V_{max}, it was apparent that both specific activities and whole body activities of coumarin, aniline, and 7-methoxycoumarin metabolism in PM were considerably lower than those in SM. Furthermore, the extent of differences between whole body activities in PM and SM were much higher than for the specific activity (Figure 4.8).

4.6.6 Intrinsic Clearance

For all the selected substrates, naïve gophers tended to have higher intrinsic clearance than the gophers previously exposed to toxins. Considering exposed and naïve gophers as population, 7-MC had the highest intrinsic clearance, which was significantly different from the rest of the substrates. NMA had the lowest intrinsic clearance (Table 4.7).

Table 4.1. Michaelis-Menten parameter estimates for aromatic hydroxylation using coumarin as substrate in hepatic microsomal suspensions prepared from naïve and toxin exposed male and female gophers (Mean ± SE) (n=6).

	Coumarin		
	K_m (μM)	V_{max} (nmol/nmol CYP450 /min)	V_{max} (nmol/100g BW/min)
Females Naïve	24.98 ± 16.9[a]	0.30 ± 0.13[a]	9.80 ± 4.91[a]
Females Exposed	16.24 ± 9.38[a]	0.17 ± 0.06[a]	10.11 ± 4.51[a]
Males Naïve	16.31 ± 6.47[a]	0.36 ± 0.20[a]	11.16 ± 5.64[a]
Males Exposed	9.37 ± 4.49[a]	0.30 ± 0.06[a]	15.29 ± 3.57[a]
ANOVA (gender)	*P=0.475*	*P=0.44*	*P=0.50*
ANOVA (status)	*P=0.473*	*P=0.47*	*P=0.64*
ANOVA (gender x status)	*P=0.92*	*P=0.77*	*P=0.69*

The values are means of six animals (SE=standard error). Means were compared using Fisher's LSD, α=0.05. Means with the same letters are not significantly different.

Table 4.2. Michaelis-Menten parameter estimates for aromatic hydroxylation using aniline as substrate in hepatic microsomal suspensions prepared from naïve and toxin exposed male and female gophers (Mean ± SE) (n=6).

	Aniline		
	K_m (μM)	V_{max} (nmol/nmol CYP450 /min)	V_{max} (nmol/100g BW/min)
Females Naïve	43.59 ± 25.42^{a}	0.95 ± 0.21^{ac}	28.67 ± 8.16^{a}
Females Exposed	24.69 ± 6.92^{a}	0.45 ± 0.16^{a}	26.79 ± 10.16^{a}
Males Naïve	34.78 ± 2.38^{a}	1.51 ± 0.24^{b}	51.74 ± 10.71^{a}
Males Exposed	38.51 ± 5.13^{a}	1.06 ± 0.13^{bc}	54.86 ± 9.83^{a}
ANOVA (gender)	*P=0.85*	*P<0.005*	*P<0.02*
ANOVA (status)	*P=0.58*	*P<0.02*	*P=0.95*
ANOVA (gender x status)	*P=0.41*	*P=0.88*	*P=0.80*

The values are means of six animals (SE=standard error). Means were compared using Fisher's LSD, α=0.05. Means with the same letters are not significantly different.

Table 4.3. Michaelis-Menten parameter estimates for N-demethylation using N-methylaniline as substrate in hepatic microsomal suspensions prepared from naïve and toxin exposed male and female gophers (Mean ± SE) (n=6).

	N-methylaniline		
	K_m (μM)	V_{max} (nmol/nmol CYP450/min)	V_{max} (nmol/100g BW/min)
Females Naïve	372.78 ± 59.71^{a}	2.81 ± 0.36^{ab}	84.09 ± 15.47^{a}
Females Exposed	295.88 ± 46.87^{ab}	2.31 ± 0.43^{ab}	113.20 ± 14.23^{a}
Males Naïve	275.52 ± 25.79^{ab}	3.77 ± 0.88^{a}	135.41 ± 42.51^{a}
Males Exposed	191.29 ± 25.75^{b}	1.25 ± 0.21^{b}	65.82 ± 14.96^{a}
ANOVA (gender)	*P<0.03*	*P=0.92*	*P=0.94*
ANOVA (status)	*P=0.07*	*P<0.02*	*P=0.43*
ANOVA (gender x status)	*P=0.93*	*P=0.07*	*P=0.06*

The values are means of six animals (SE= standard error). Means were compared using Fisher's LSD, α=0.05. Means with the same letters are not significantly different.

Table 4.4. Michaelis-Menten parameter estimates for N-demethylation using N,N-dimethylaniline as substrate in hepatic microsomal suspensions prepared from naïve and toxin exposed male and female gophers (Mean ± SE) (n=6).

	N,N-dimethylaniline		
	K_m (μM)	V_{max} (nmol/nmol CYP450 /min)	V_{max} (nmol/100g BW/min)
Females Naïve	92.30 ± 14.33^{a}	3.31 ± 0.57^{ab}	97.63 ± 23.27^{a}
Females Exposed	151.41 ± 15.46^{b}	3.59 ± 0.45^{ab}	191.72 ± 37.63^{a}
Males Naïve	95.67 ± 9.01^{a}	4.97 ± 0.84^{a}	178.42 ± 49.93^{a}
Males Exposed	101.28 ± 15.81^{a}	2.55 ± 0.20^{b}	129.33 ± 16.44^{a}
ANOVA (gender)	*P=0.109*	*P=0.59*	*P=0.79*
ANOVA (status)	*P<0.04*	*P=0.07*	*P=0.52*
ANOVA (gender x status)	*P=0.07*	*P=0.03*	*P=0.05*

The values are means of six animals (SE=standard error). Means were compared using Fisher's LSD, α= 0.05. Means with the same letters are not significantly different.

Table 4.5. Michaelis-Menten parameter estimates for O-demethylation using 7-Methoxycoumarin as substrate in hepatic microsomal suspensions prepared from naïve and toxin exposed male and female gophers (Mean ± SE) (n=6).

	7-Methoxycoumarin		
	K_m (μM)	V_{max} (nmol/nmol CYP450 /min)	V_{max} (nmol/100g BW/min)
Females Naïve	3.59 ± 0.58[a]	3.43 ± 1.26[ab]	110.70 ± 46.77[a]
Females Exposed	4.85 ± 2.02[a]	2.83 ± 1.15[a]	173.15 ± 74.05[a]
Males Naïve	4.12 ± 0.50[a]	7.06 ± 2.12[b]	269.74 ± 105.84[a]
Males Exposed	3.18 ± 0.33[a]	4.09 ± 0.46[ab]	207.38 ± 28.46[a]
ANOVA (gender)	*P=0.61*	*P=0.09*	*P=0.18*
ANOVA (status)	*P=0.89*	*P=0.21*	*P=0.99*
ANOVA (gender x status)	*P=0.32*	*P=0.40*	*P=0.38*

The values are means of six animals (SE= standard error). Means were compared using Fisher's LSD, α= 0.05. Means with the same letters are not significantly different.

Table 4.6. Comparison of cytochrome P450 kinetics for different substrates: affinity, specific activity and whole body activity in exposed and naïve gophers (Mean ± SE).

	K_m(μM)		V_{max}(nmol/nmolCYP450/min)		V_{max} (nmol/100g BW/min)	
Substrates	Exposed	Naïve	Exposed	Naïve	Exposed	Naïve
Coumarin	13.00±5.05^{a}	20.65±8.74^{a}	0.23±0.05^{a}	0.33±0.11^{a}	12.70±2.85^{a}	10.48±3.57^{a}
Aniline	31.60±4.61^{a}	39.19±12.24^{a}	0.75±0.13^{a}	1.23±0.17^{a}	40.83±7.96^{ab}	40.21±7.30^{ab}
NMA	243.58±29.98^{b}	324.15±34.30^{b}	1.78±0.28^{c}	3.29±0.48^{b}	89.51±12.16^{b}	109.75±22.91^{bc}
DMA	126.35±12.97^{c}	93.98±8.08^{c}	3.07±0.28^{b}	4.14±0.55^{bc}	160.53±21.72^{c}	138.02±28.95^{c}
7-MC	4.01±1.01^{a}	3.85±0.37^{a}	3.46±0.62^{b}	5.24±1.30^{c}	190.26±38.17^{c}	190.22±60.15^{c}

The values are means of 12 animals (SE=standard error). Means were compared using Fisher's LSD, α= 0.05. Means with the same letters are not significantly different.

Table 4.7. Comparison of microsomal intrinsic clearance values analyzed by enzyme kinetic method: $Cl_{int} = V_{max} / K_m$ for selected compound between naïve gophers and gophers exposed previously to toxins.

Intrinsic clearance (ml/min/g protein)			
Substrates	Exposed n = 12	Naïve n = 12	Exposed and Naïve as population n = 24
Coumarin	31.40 ± 7.86[a]	37.24 ± 16.99[a]	34.32 ± 9.18[a]
Aniline	22.72 ± 4.54[a]	29.42 ± 4.38[a]	26.07 ± 3.17[a]
NMA	6.34 ± 0.56[a]	8.10 ± 1.48[a]	7.22 ± 0.80[a]
DMA	22.42 ± 1.81[a]	35.05 ± 6.22[a]	28.73 ± 3.43[a]
7-MC	977.35 ± 172.18[a]	978.83 ± 207.47[a]	978.09 ± 131.84[b]

SE=standard error. Means were compared using Fisher's LSD, $\alpha = 0.05$. Means with the same letters are not significantly different.

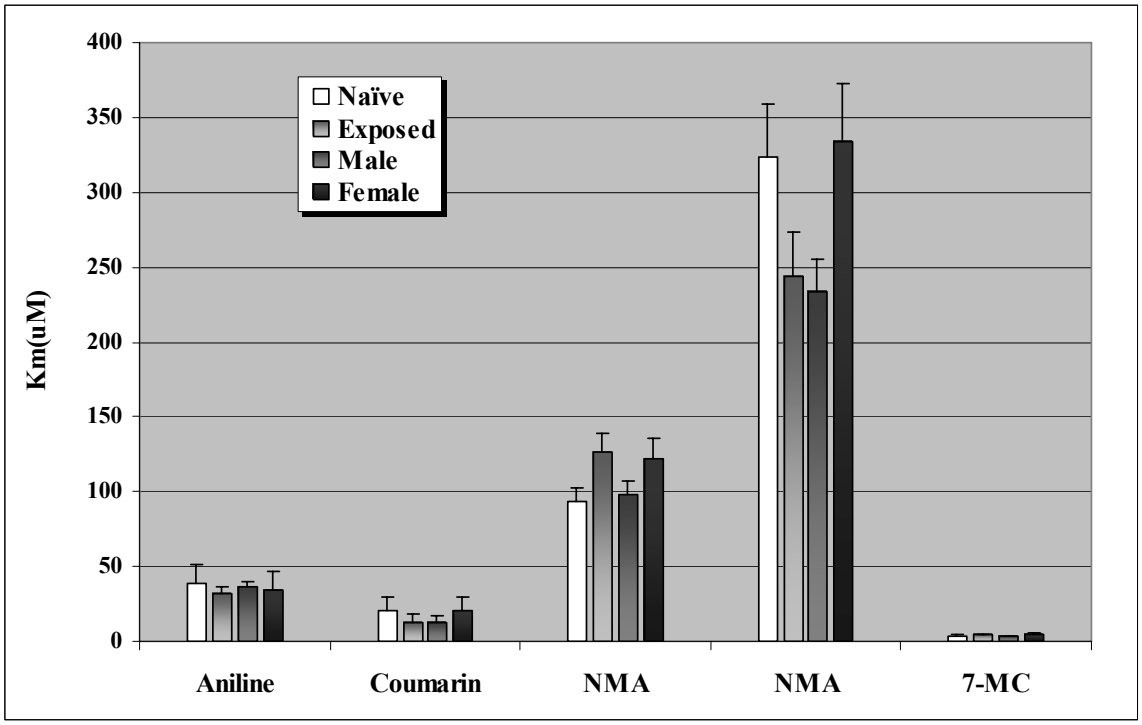

Figure 4.4. Comparison of cytochrome P450 enzyme K_m values for different substrates: N-methylaniline has the highest K_m values, whereas, 7-methoxycoumarin has the lowest K_m values regardless of status and gender differences. N-demethylation reactions have higher K_m than other reactions.

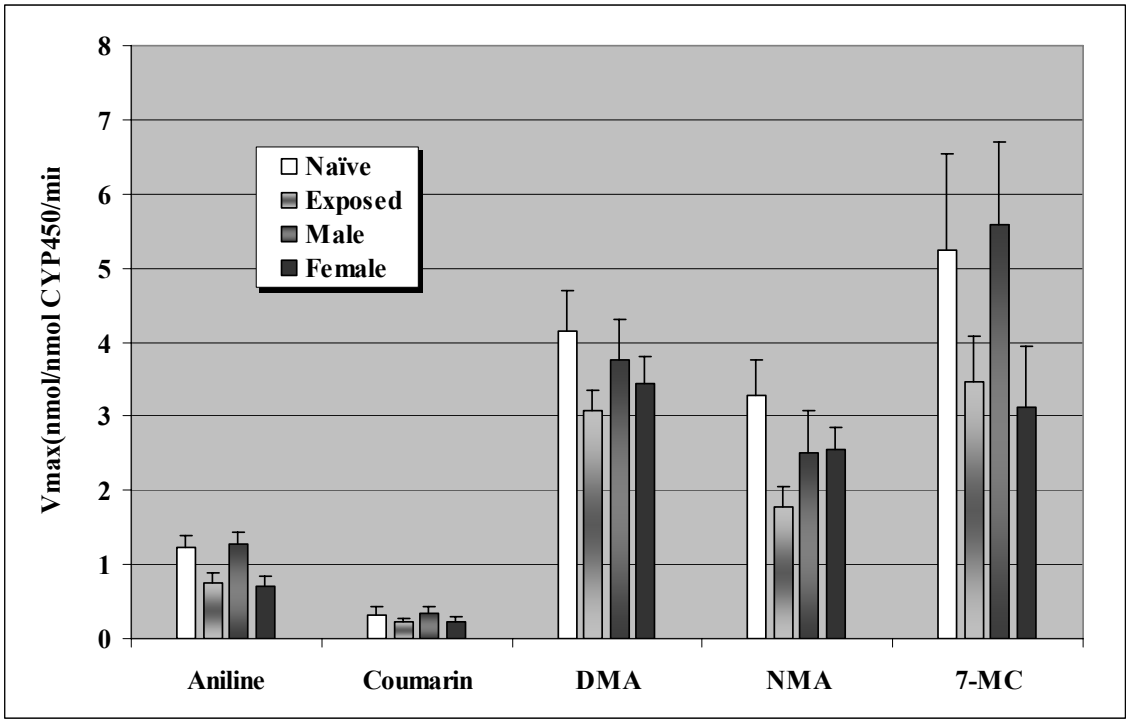

Figure 4.5. Comparison of cytochrome P450 specific activity for different substrates: 7-methoxycoumarin has the highest specific activity, whereas, coumarin has the lowest specific activity. Generally, demethylation reactions have higher specific activities than aromatic hydroxylation.

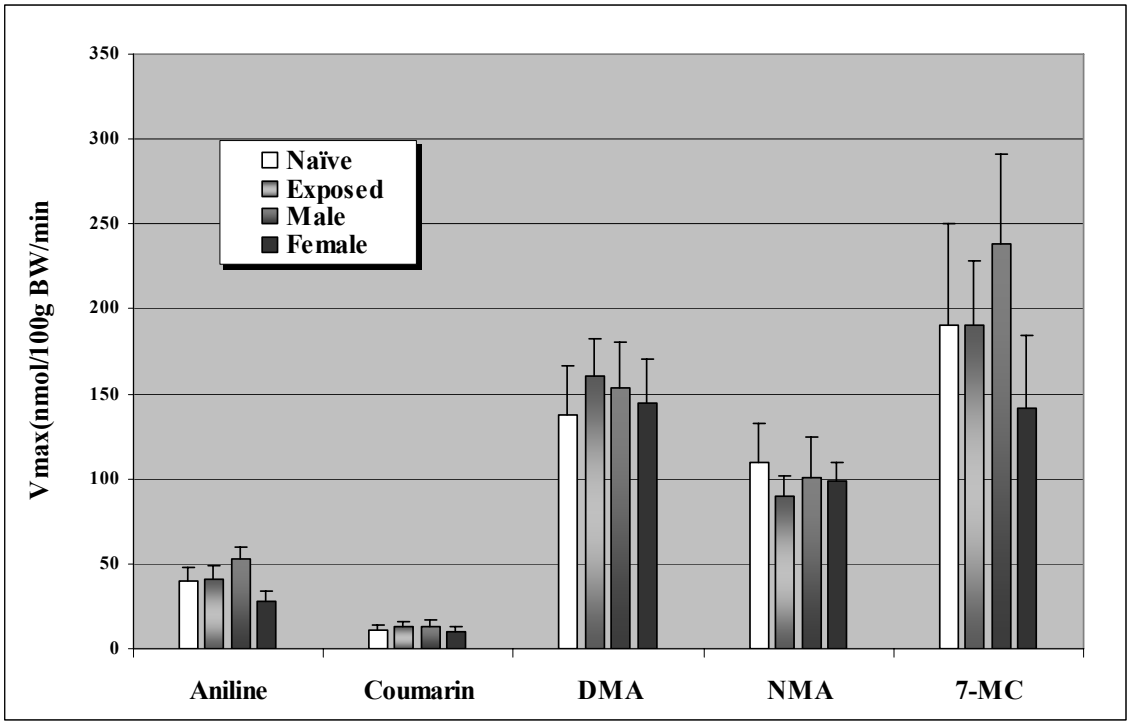

Figure 4.6. Comparison of cytochrome P450 whole body activity for different substrates. The trend of whole body activity is the same as the specific activity.

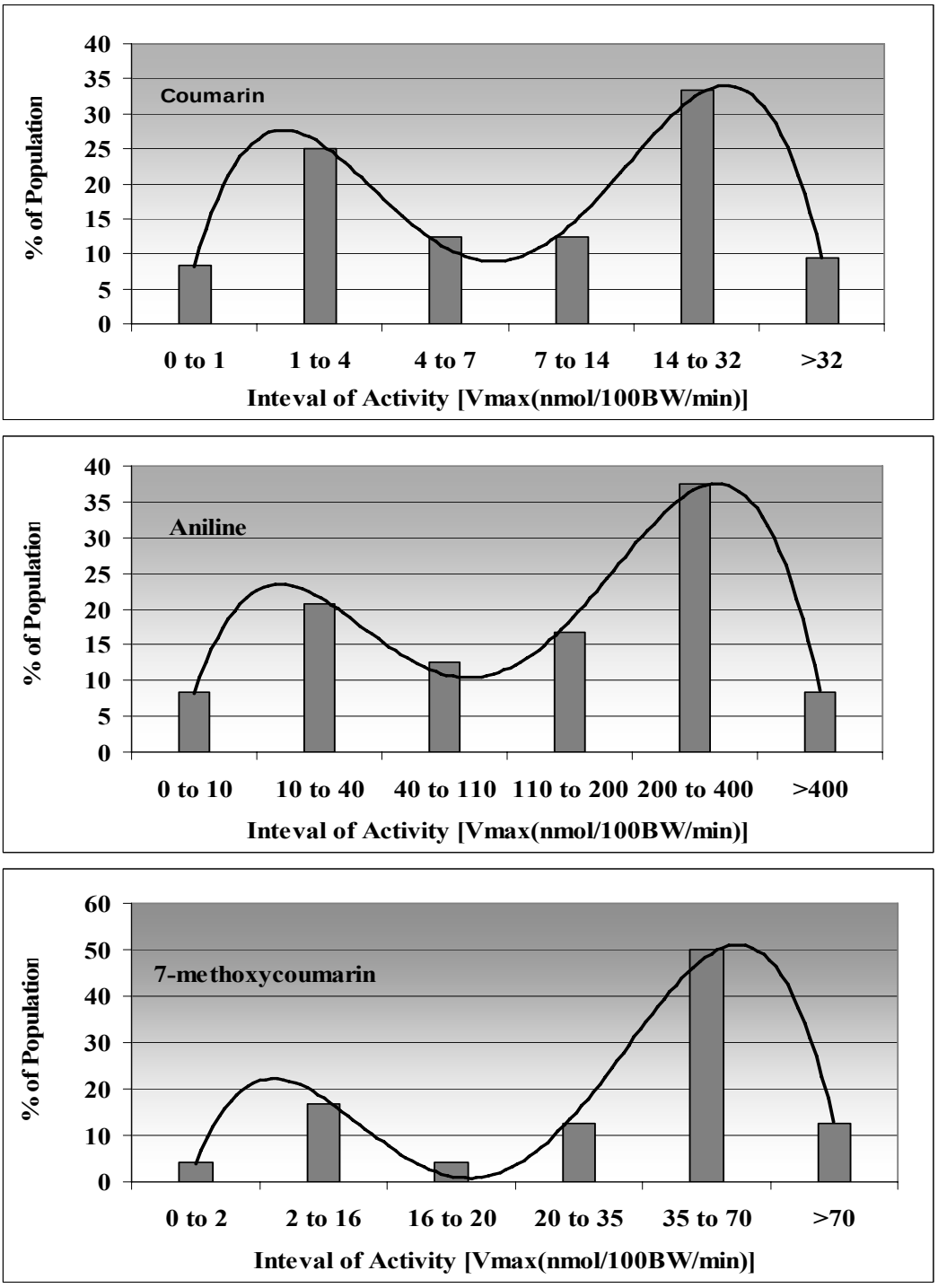

Figure 4.7. Identifiable subgroups in the population of 24 gophers with distinctly different enzyme characteristics. The respective subgroups of gophers were characterized either as poor metabolizers or strong metabolizers based on their whole body activities.

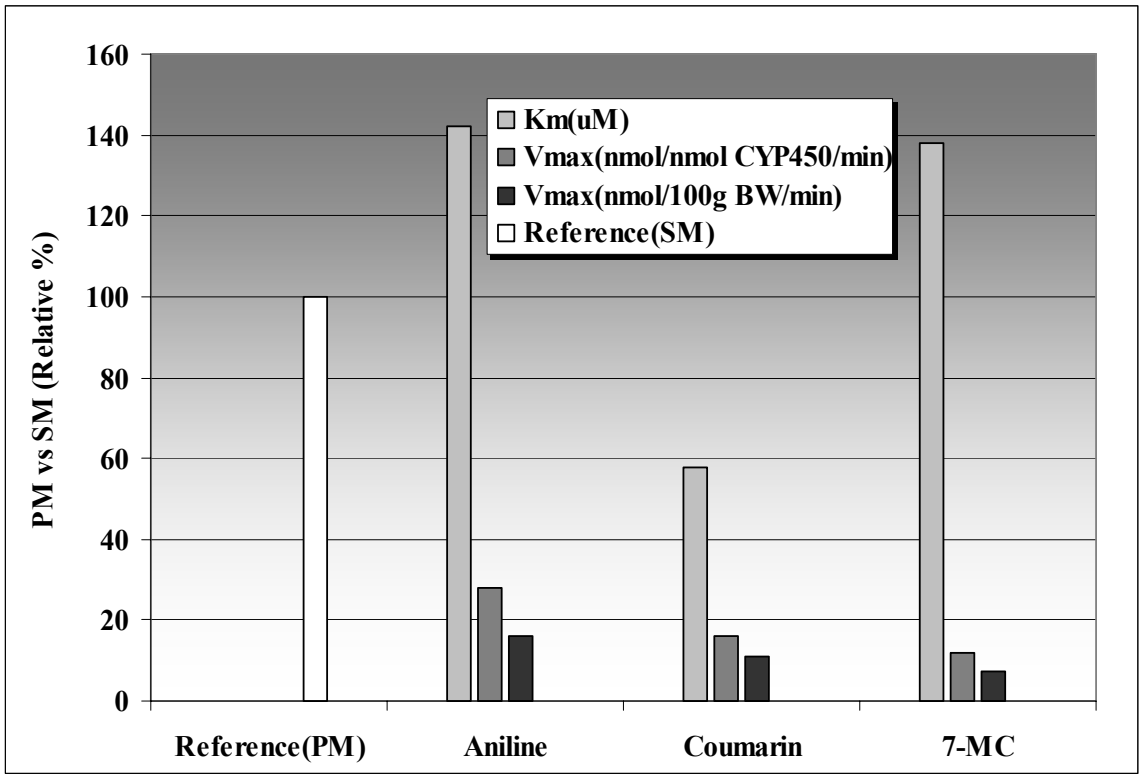

Figure 4.8. Relative differences in K_m and V_{max} between poor metabolizer (PM) and strong metabolizer (SM). White bar on the graph indicates K_m and V_{max} for the SM (100%), which is then compared to relative values for K_m (grey bars) and V_{max} (dark grey bars for specific activity and black bars for whole body activity).

4.7 Discussion

In many species, hepatic xenobiotic biotransformation involving the cytochrome P450 enzyme system can be influenced by both sex and toxin exposure status (MacLeod et al., 1987; Loi and Vestal, 1988). However, no scientific information is available for gophers. In this study three different cytochrome P450 detoxification pathways, aromatic hydroxylation, N-demethylation and O-demethylation, were investigated in gophers to assess the influence of gender and toxin exposure status. In general, the present study demonstrated that naïve male gophers have considerably higher specific enzyme activities than naïve females for a given biotransformation pathway, although these differences were not statistically significant. This difference was also apparent when the results were expressed on the basis of whole body activity. The data suggest that naïve male gophers likely have higher rates of xenobiotic metabolism *in vivo* than naïve females. Chapter 3 indicated that naïve female gophers had 16.8 % less total cytochrome P450 content than naïve male gophers. This difference in total cytochrome P450 content may help to explain why naïve female gophers metabolize selected compounds at a different rate than naïve male gophers.

The observed differences associated with gender in the present study are consistent with the findings in other species. Sex dependent differences in biotransformation of chemicals have been found in other rodent species (Schenkman and Greim, 1993). For instance, male rats have been found to have higher rates of xenobiotic metabolism than female rats (Kedderis and Mugford, 1998).

Exposure status is another factor that influenced the activity of cytochrome P450 enzymes in gophers. For all the selected substrates, naïve gophers were found to have higher specific activities than exposed gophers. However when whole body activity was calculated, there was almost no difference between naïve gophers and exposed gophers (Table 4.6). This may be the result of exposure to certain compounds, which exist in nature or from toxins used to control the gopher population, which can function as cytochrome P450 inhibitors. Such inhibitors can reduce the activity of enzymes responsible for the metabolism of selected compounds in this study However, the

effects of exposure status appeared to be influenced by sex in gophers. Females exhibited a more marked decrease in specific enzyme activity (40-50%) than males (20%).

Exposed gophers demonstrated higher total cytochrome P450 enzyme contents yet exhibited lower specific activities, which resulted in reduced differences of whole body activities between naïve and exposed gophers. This may indicate that when gophers are exposed to toxins, the activities of detoxification enzymes decrease, whereas in exposed gophers the enzymes content increased, which can over time compensate for the total detoxification capacity in this group of gophers. However, upon examining Table 4.6, it can be inferred that there are subtle differences between naïve and exposed gopher groups for aniline, coumarin, and 7-methoxycoumarin, and the exposed gophers have slightly higher activity than naïve for N,N-dimethylaniline metabolism.

In addition to the variations in enzyme content, interindividual variability involving enzyme induction or inhibition has also been identified in humans (Lin and Lu, 1998) and other species (Pampori and Shapiro, 1999). Gophers also likely exhibit such interindividual variation in present study. However, the extent of hepatic cytochrome P450 inhibition or induction of female and male gophers can be different due to various factors: 1) animal factors: sex specific isozymes (Pampori and Shapiro, 1999); and 2) environmental factors: different components in diet (Yang et al., 1992). Furthermore, certain compounds can be an inducer for certain isozymes as well as an inhibitor for other isozymes simultaneously (Klassen, 2001). This may explain why exposed female gophers have slightly higher cytochrome P450 content than exposed males and why exposed female gophers have considerably higher enzyme activity than exposed males for N-demethylation, yet exposed male gophers have higher activity than exposed females for aromatic hydroxylation and O-demethylation.

V_{max} and K_m are the two parameters that define the kinetic behavior of an enzyme as a function of substrate concentration. K_m is an approximate measure of the affinity of the substrate for the enzyme and V_{max} is the maximum rate of an enzyme catalyzed reaction (i.e. when the enzyme is saturated by the substrate). In our study,

both two binding site model and one binding site model were tested when calculating V_{max} and K_m. The reason for this was all the selected substrates could be metabolized by more than one enzyme. However, most of the populations did not fit into two binding site model. Thus, the results in the present study were calculated by using the one binding site model. Furthermore, the ultimately fates of these substrate rather than the contribution of different enzymes were considered in our study. Thus, the results in the present study were calculated by using the one binding site model which reflected a combined effect of all enzymes responsible for the biotransformation of these compounds.

K_m values for N-methylaniline were found to be the highest among the selected substrates for both naïve and exposed gophers; whereas, K_m values for 7-MC were the lowest. As demonstrated in Figure 4.1, N-demethylation enzymes had a higher K_m value than aromatic hydroxylation enzymes. If the situation where K_m value is high, the substrate would be expected to be poorly metabolized until it reached a certain concentration. However, if the inherent toxicity of such a substrate is high, systemic toxicity can occur before the compound is metabolized at a toxicologically meaningful rate. Thus, in gophers, compounds that undergo detoxification via N-demethylation pathways would be expected to have higher potential to cause a toxic effect than compounds that are detoxified via aromatic hydroxylation pathways.

Examination of the V_{max} values revealed that coumarin aromatic hydroxylation had the lowest value in all gophers irrespective of gender or status. In fact 7-hydroxycoumarin (7-HC) was found to be the major metabolite formed by gopher microsomal preparations. This finding is consistent with most other species where coumarin is hydroxylated to 7-hydroxycoumarin, a nontoxic metabolite (Vassallo et al., 2004). The formation rate of 7-HC was in the range of low nmol per nmol cytochrome P450 per min, which was extremely low in comparison to other substrates studied here.

Notably, 7-methoxycoumarin O-demethylation had the highest V_{max} value based on total cytochrome P450 content. In addition, taking each separate reaction group into consideration the V_{max} values based on cytochrome P450 content for demethylation

reactions were found to be higher than aromatic hydroxylation reactions (Figure 4.2).

As mentioned in the rationale section (4.3), the difference found in K_m or V_{max} values for given compounds can eventually lead to different toxic outcomes. Either of these kinetic features described above can be exploited to develop novel approach to the management of gopher population. This topic will be further elaborated in chapter 8.

Of note, much of the differences in metabolism of coumarin, aniline and 7-methoxycoumarin metabolism in microsomes prepared from sampled gophers were not statistically significant. The main reason for this is that our sample size was too small relative to the large degree of interindividual variability associated with cytochrome P450 enzyme catalyzed biotransformation. Variability of catalytic activity of cytochrome P450 enzymes is a common characteristic of this enzyme system, and this is mainly associated with large range of variability in the expression of enzyme protein (Lin and Lu, 2001). This feature has been identified in the present study in gophers (for details see Chapter 3).

A large variability in catalytic activities of hepatic cytochrome P450 enzymes was confirmed in our study. In particular, high variation between individuals was noted for coumarin, aniline and 7-methoxycoumarin metabolism in gophers. Interestingly, V_{max} values varied between individuals by as much as 180 fold for coumarin, and 50 fold for aniline and 7-methoxycoumarin. Yet, there was a weak correlation between the individual cytochrome P450 content and their catalytic activity in our study. This can be explained by the fact that the activities of various metabolic pathways examined in our study were comprised of the sum of activities of different CYP450 isoforms.

Since metabolic capacity of the cytochrome P450 enzyme system was not equal in all members of the gopher population, the individuals tested for this research were divided into two subgroups: 1) poor metabolizers, representing those having considerable lower V_{max} values; and 2) stronger metabolizers, the remainder of the studied gopher population.

As a result of variation in metabolism, the metabolic conversion and excretion rate of toxins would be expected to vary among individuals. This is normal for the xenobiotic metabolism catalyzed by cytochrome P450 system (McKinnon and Evans 2000). In our study, it was apparent that both specific activities and whole body activities of coumarin, aniline, and 7-methoxycoumarin metabolism in PM were considerably lower than in SM. Furthermore, the extent of difference between whole body activities in PM and SM was much higher than the specific activity, and this feature has practical implications. The reason for this is that the most common approach is to calculate the dosage according to the body weight or body surface rather than the specific activity of the detoxification enzymes. Therefore the knowledge of specific metabolic characteristics in gophers will be advantageous for developing a potent gopher control method. This issue will be further addressed in chapter 8.

Another important parameter that will determine the toxicity of a given compound is systemic clearance (Cotreau et al., 2005). To extrapolate the *in vitro* kinetic data to metabolic activity *in vivo*, the concept of intrinsic clearance (CL_{int}) is very useful and important (Lin, 1998). The reason for this is liver microsomal intrinsic clearance values can be scaled and used to predict hepatic clearance (Lin, 1998). For compounds with low intrinsic clearance values, the elimination rate of the compounds depends on enzymatic activity. This means enhancement in hepatic enzyme activity, resulting from the addition of an inducer, will have a more profound effect on clearance of such compounds. Conversely, enzyme inhibitors will have the opposite effect. On the other hand, for compounds with a high intrinsic clearance, increased enzyme activity will have little effect on hepatic clearance (Labaune, 1989). However, our ultimate purpose is to design a specific toxicant which will be delivered to the gophers by the oral route. Considering the fact that oral clearance depends on intrinsic clearance and plasma protein binding, induction and inhibition will influence the oral clearance of any compound whether it has a high CL_{int} or low CL_{int} when it is administered by the oral route.

Clearance, when combined with the volume of distribution, determines the half-life of a compound; whereas, intrinsic clearance, when combined with absorption,

determines the oral bioavailability of a compound. Half-life and oral bioavailability are key determinants of duration and extent of the toxicity. As such the bioavailability of 7-methoxycoumarin is expected to considerably increase when the enzyme activity is inhibited, since it has the highest intrinsic activity. However, the bioavailability of N-methylaniline will not change appreciably since its intrinsic activity is the lowest in all these selected compounds. As mentioned previously, the enhancement in half life and bioavailability of a compound will result in the increasing toxic potency of the parent compound, considering the inherent toxicity of such compound. Thus, co-administration of inhibitors with toxin will be a desirable method for gopher control. This will be further discussed in chapter 8.

In summary, the results of the present studies indicate that, when gophers are exposed to toxins, the activity of the enzymes decreased, but they can make up for the decreased activity of the enzymes by enhancing the content of enzymes. The study identified a noteworthy inter-individual variation existing in the CYP450-mediated metabolism of some substrates including coumarin, aniline, and 7-methoxycoumarin but, there were no significant differences for the intrinsic clearance between naïve gophers and exposed gophers for all selected substrates. Nonetheless, as evidenced from our field observations, the gophers appear to cope with the toxins used for their control, since the densities of gophers were similar on both fields. However, considering that the gophers harvested from the field where chemical control was used had higher content of cytochrome P450 in their livers, it can be inferred that failure to decrease the gopher population on that field was most likely associated with an increased metabolic potential of detoxification in these animals. Therefore, in essence, our data support the principle that animals exposed to toxins increase cytochrome P450 content, and thus acquire resistance.

5.0 STUDY ON INHIBITION OF SELECTED DETOXIFICATION PATHWAYS

5.1 Introduction

Numerous compounds may alter the activity of cytochrome P450 system with the net result being either enhancement or inhibition of the metabolism of other compounds. The mechanisms of cytochrome P450 inhibition can be generally divided into reversible inhibition and irreversible inhibition.

Reversible inhibition is the most common mechanism, which occurs as a result of direct competition for a binding site on a cytochrome P450 enzyme between a substrate and an inhibitor. A reversible inhibitor that binds to the enzyme active site in place of the substrate is described as a competitive inhibitor, whereas, a reversible inhibitor that binds to the enzyme at a site other than the active site is described as a noncompetitive inhibitor, which has no effect on substrate binding (Yan and Caldwell, 2001). In both situations, the cytochrome P450 enzymes return to normal function after the inhibitor has been eliminated from the body. In contrast, irreversible inhibition occurs when certain compounds or its metabolites bind to the enzyme and cause irreversible loss of function, which persists even after elimination of the inhibitor. Thus, the functional activity can only be restored by synthesis of new enzymes, which may take several days (Meyer and Rodvold, 1996; Lin and Lu, 2001).

One of the most potent classes of cytochrome P450 inhibitors are imidazoles (Turan et al., 2001; Wilkinson et al., 1972). These inhibitors are N-substituted imidazole derivatives and have a nitrogen atom with sterically accessible nonbonded electrons (Wilkinson et al., 1974; Mailman et al., 1974) that carry the potential to interact with microsomes binding tightly with the prosthetic heme iron of cytochrome P450 proteins. Because the heme prosthetic group is the oxidation center for cytochrome P450

catalyzed reactions, compounds with lone electron pairs tend to be potential competitive cytochrome P450 inhibitors.

The principal mechanism of action for imidazole derivatives is to impair the integrity of fungal cell membranes by inhibiting the biosynthesis of membrane lipids through the inhibition of the cytochrome P450-dependent 14α-demethylase activity (Van den Bossche et al., 1983; Bodey, 1992). On the molecular basis clotrimazole (CTZ) is a potent specific inhibitor of cytochrome P450 enzymes, which interferes with the cytochrome P450 dependent 14-α-demethylation of lanosterole or 24-methylendihydrolanosterole, and finally destroys fungal cellular integrity. Clotrimazole is recognized as potent ligand of the heme iron atom of cytochrome P450 (Zhang et al., 2002). Furthermore, in human liver microsomes clotrimazole was shown to potently inhibit CYP2A6-mediated coumarin 7-hydroxylation (K_i 0.42 μM) (Draper et al., 1997). Although clotrimazole appears to be a strong inducer of human CYP3A (Ritter and Franklin, 1987), this compound is unlikely to behave as such in a clinical situation. The reason for this is its inhibitory effect should overcome inducing effects.

The problems associated with the increasing populations of the Richardson's ground squirrels in Saskatchewan (Saskatchewan "Gophers" Survey 2001) and the need for better alternative control method for this species were introduced in the previous chapters. The previous study demonstrated that cytochrome P450 enzyme system components are much higher in the gophers previously exposed to toxic baits than the naïve gophers even though the specific activity of the enzyme system based on cytochrome P450 enzyme content in the exposed gophers is lower than the activity in the naïve gopher. Therefore, hepatic cytochrome P450 enzymes play a major role in the detoxification of toxins used to control population of gophers. As such, finding a potent inhibitor of the cytochrome P450 enzymes in gophers would be valuable. The inhibition of detoxification enzymes would result in increasing toxicity of a compound with inherent toxicity; thus, it would increase the number of options that are available in designing a species-specific method to control the gopher population.

In this study, the inhibitory potential of clotrimazole (Figure 5.1) on different cytochrome P450 catalyzed reactions was evaluated in gopher hepatic microsomes. These studied reactions included aromatic hydroxylation using aniline, N-demethylation using N-methylaniline and N,N-dimethylaniline, and O-demethylation using 7-methoxycoumarin as probe substrate.

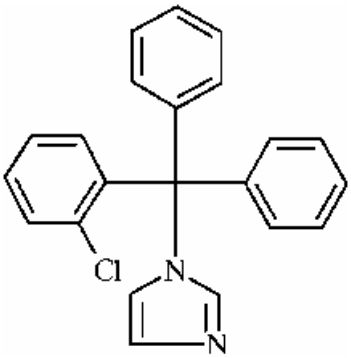

Figure 5.1. Chemical structure of clotrimazole. Clotrimazole in our experiment was purchased from Sigma (Sigma, St Louis, Missouri, U.S.A.).

5.2 Materials and Methods

5.2.1 Inhibition procedure

Gophers, preparation of liver microsomes, assay of cytochrome P450 enzyme activity and optimal analytical techniques were as previously described. The inhibition of cytochrome P450 reactions were investigated using the standard assay condition for each cytochrome P450 substrate. The concentration for the substrate in the final reaction mixture used was 20μg/ml for dimethylaniline, 50μg/ml for methylaniline, 50μg/ml for aniline, 5μg/ml for 7-methoxycoumarin. Substrate concentrations for all these compounds were chosen as approaching the Km value for these substrates determined in the gopher liver microsomes described in Chapter 4. Incubations of the assays were performed in the presence of a range of inhibitor (CTZ) concentrations prepared from stock solution of CTZ dissolved in methanol at a concentration of 2mM. The concentration of CTZ was between 0.0001μM to 100μM in the final reaction mixture.

There was no visible evidence of precipitation of any of these compounds over the concentration range studied.

The optimal reaction conditions were developed for all substrates as described in Chapter 4. The reaction mixtures containing microsome preparation, substrate, and CTZ were pre-incubated at 37°C for 10 min. Blank samples with no substrate and with the highest CTZ concentration in the absence of NADPH were prepared under the same condition as the assay samples. The reaction was initiated by addition of 5 µl of 10 mM NADPH (final concentration 0.5 µM) and incubated in a water bath (30 min for aniline, 5 min for the rest of the substrates) at 37°C. The reaction was terminated by addition of 100µl of acetonitrile.

5.2.2 HPLC analysis

The reaction mixture was centrifuged at 14,000 rpm for 30min. The aqueous phase was then filtered through a 0.45 µm syringe filter, and 10 µl was injected into the HPLC. For the selected substrates of interest, optimal reaction and HPLC analysis conditions were developed through validation of the method (for details see Chapter 4). These selected substrates and their metabolites were separated using a reversed-phase column (symmetry column: silica-based C_{18} (2), LUNA, 150 x 4.60mm, 3µm, Phenomenex, Torrance, CA) and a HPLC system with autoinjector (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) and fluorescence detector (RF-551, Spectrofluorometric detector, SHIMADZU, Columbia, MD, USA).

The inhibition constant (IC_{50}) was used to define the interactions between the inhibitor and cytochrome P450. IC_{50} values represent the concentration of the inhibitor that was required for 50% inhibition of an enzyme reaction. GraphPad Prism 3.03 software for Windows (GraphPad Software, San Diego, CA) was used for data analysis. Both one site and two sites competition models were tested. Finally, it was decided that IC_{50} values should be determined by using the one site competition model.

5.3.3 Statistical Analyses

All data are presented as means ± standard error (SE). Statistical analyses were carried out using ANOVA from the microcomputer package Number Cruncher Statistical System (HINTZE, 1995). The means were compared using Fisher's LSD test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

5.3 Results

5.3.1 Procedural

Different inhibitor concentrations were tested. A nonlinear regression analysis of the data using different inhibition models was conducted with GraphPad 3.03. For each set of reactions seven points of CTZ concentrations were chosen from the best fit curve to calculate IC_{50} values (Figure 5.2).

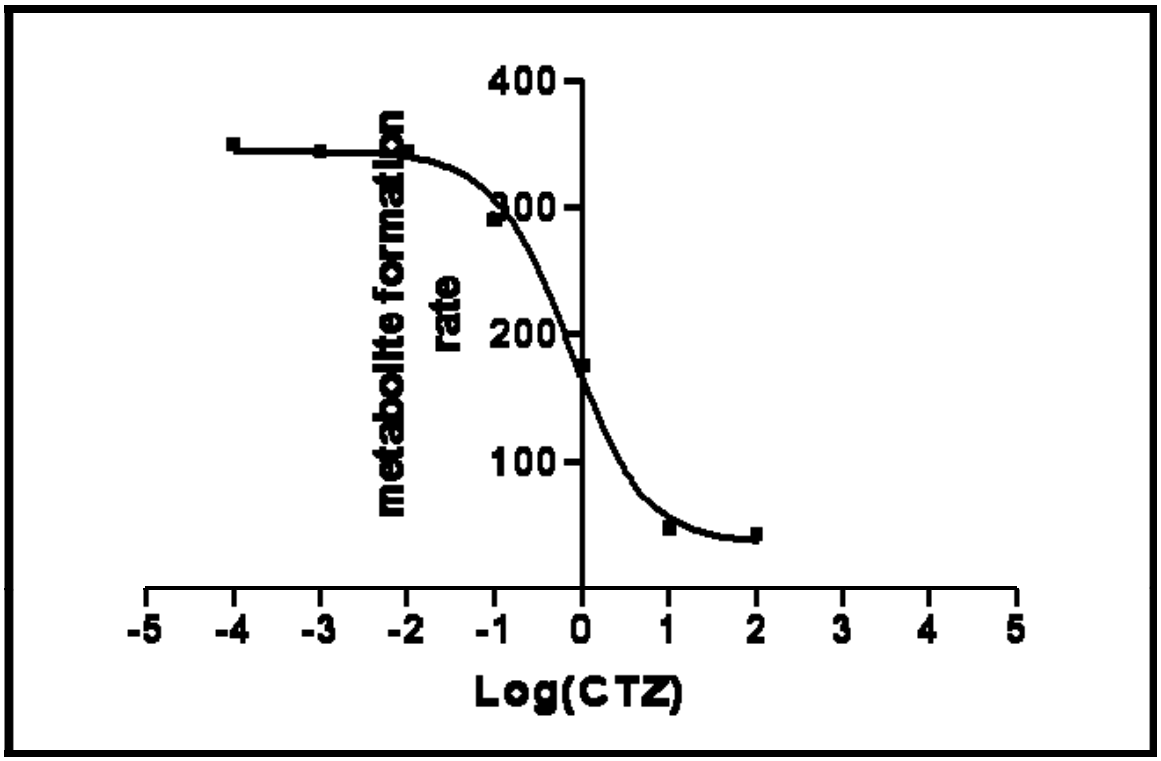

Figure 5.2. Effect of CTZ on gopher hepatic microsomal cytochrome P450 enzyme activity expressed as metabolite formation rate where control contains no inhibitor. Seven different CTZ concentrations were used to calculate the IC_{50} of CTZ to different cytochrome catalyzed biotransformation pathways. The metabolite of each substrate was measured by HPLC methods described in the previous chapter.

5.4.2 Experimental

The results of inhibition reactions for all substates tested in the present study are presented in Table 5.1. The characteristics of inhibition in terms of IC_{50} values varied between different substrates.

5.4.2.1 N-methylaniline inhibition

There were no statistically significant differences in IC_{50} values with respect to gender or status for N-methylaniline metabolism (Table 5.1). However, IC_{50} values of naïve gophers for both females and males were lower than exposed gophers. Female gophers tended to have higher IC_{50} values than male gophers within naïve and exposed categories. Thus, for N-methylaniline metabolism, clotrimazole appeared to be more potent inhibitor in exposed gophers than to naïve gophers.

5.4.2.2 N,N-dimethylaniline inhibition

There were no statistically significant differences in IC_{50} values with respect to both gender and status for N,N-dimethylaniline metabolism. However, there were still trends were found for the inhibitory potency of clotrimazole. IC_{50} values of naïve gophers tended to be lower than exposed gophers for both females and males, which tended to be higher for males than for females for both naïve and exposed gopher groups.

5.4.2.3 7-methoxycoumarin inhibition

There were statistically significant differences for IC_{50} values for 7-methoxycoumarin inhibition between females and males within the group of exposed gophers. Considering both naïve and exposed gophers as one population, there was also a statistically significant difference for IC_{50} values between genders where male gophers had higher IC_{50} values than female gophers ($P<0.02$).

5.4.2.4 Aniline inhibition

There were no statistically significant differences in IC_{50} values between naïve and exposed gophers of the same gender for aniline inhibition. However, the IC_{50} values for female exposed gophers were higher than exposed and naïve males and this difference were statistically significant. Furthermore considering both naïve and exposed gophers as one group, there were statistically significant differences for IC_{50} values between gender; female gophers had higher IC_{50} values than male gophers ($P<0.03$). Also, the IC_{50} values of exposed gophers were found to be higher than naïve gophers for both sexes (Figure 5.4).

Table 5.1. Inhibition of cytochrome P450 enzyme reactions by clotrimazole in hepatic microsomal preparations from gophers. Data represent the mean ± SE from 6 gophers.

Subject of Study	IC_{50} (μM)			
	NMA	**DMA**	**7-MC**	**Aniline**
Females Naïve	0.24 ± 0.07^{a}	0.34 ± 0.20^{a}	0.39 ± 0.13^{ab}	2.10 ± 2.06^{ab}
Females Exposed	0.21 ± 0.03^{a}	0.57 ± 0.28^{a}	0.48 ± 0.08^{a}	3.78 ± 2.21^{a}
Males Naïve	0.22 ± 0.13^{a}	0.67 ± 0.24^{a}	0.79 ± 0.09^{ab}	0.27 ± 0.14^{b}
Males Exposed	0.17 ± 0.06^{a}	0.71 ± 0.25^{a}	0.76 ± 0.18^{b}	0.45 ± 0.18^{b}
ANOVA (gender)	*P=0.74*	*P=0.72*	*P<0.02*	*P<0.03*
ANOVA (status)	*P=0.63*	*P=0.33*	*P=0.83*	*P=0.49*
ANOVA (gender x status)	*P=0.91*	*P=0.40*	*P=0.64*	*P=0.39*

The values are means of six animals, SE=standard error. Means were compared using Fisher's LSD, $\alpha = 0.05$. Means with the same letters are not significantly different.

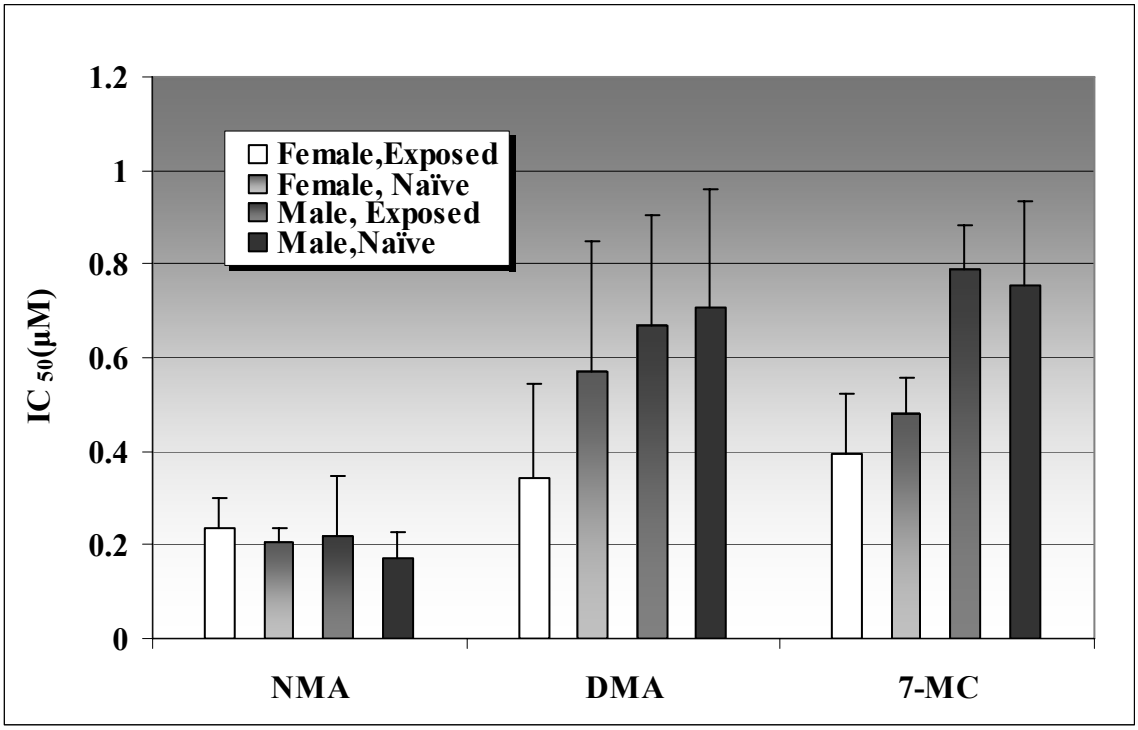

Figure 5.3. IC_{50} of CTZ for different cytochrome P450 catalyzed reactions including N-demethylation of N-methylaniline and N,N-dimethylaniline and O-demethylation of 7-methoxycoumarin in four groups of gopher hepatic microsomal preparations from gophers (n=6).

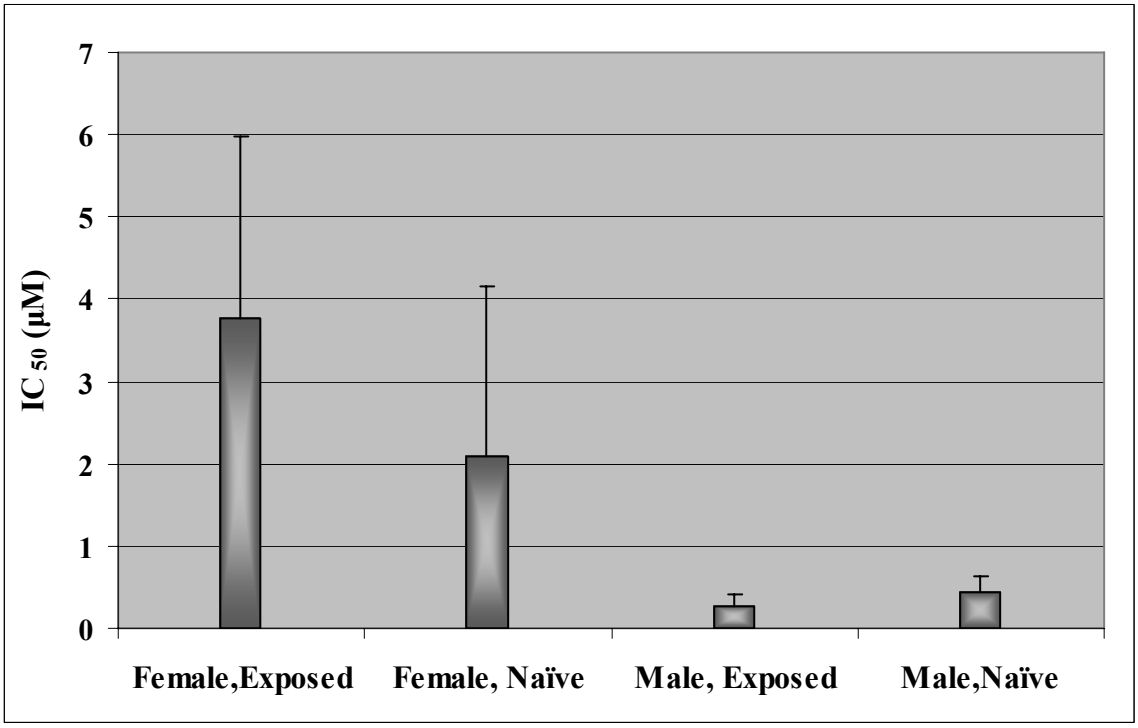

Figure 5.4. IC_{50} of CTZ for cytochrome P450 catalyzed aromatic hydroxylation of aniline in the four groups of gopher hepatic microsomal preparations (n=6).

5.4 Discussion

Identification of compounds that inhibit the cytochrome P450 enzymes in gophers increases the number of options available for designing more efficient species-specific methods to control the gopher population.

As discussed in Chapter 4, the inhibition of enzymes may result in a considerable enhancement in the bioavailability of a toxin. Especially, high clearance compounds are subject to extensive first-pass metabolism and are more sensitive to cytochrome inhibition than low clearance compounds following oral exposures. Thus, the oral co-administration of a high clearance compound with a potent inhibitor produces a synergistic effect by increasing bioavailability through the inhibition of first-pass metabolism and a reduction in the oral clearance. Whereas, for a low clearance compounds, first-pass metabolism is minor and a significant inhibitory effect is only seen in hepatic or systemic clearance (Lin and Lu, 2001). Inhibition of systemic clearance would lead to enhanced exposure to the toxin. Therefore, it is important to acknowledge that the favorable consequences of enzyme inhibition as a gopher control strategy should always be a reduction in hepatic metabolism and an increase in bioavailability when the parent compound exerts the toxicity. Bioavailability is defined as a measurement of the rate and extent of an active compound that reaches systemic circulation and is available at the site of action (Shargel and Yu, 1999). Consequently, bioavailability is an important determinant of systemic exposure and knowledge of a compound's bioavailability will help to determine the toxicity of selected compound in the target animals (gophers). In addition to this, the synergistic effect of a potent toxin and a potent cytochrome P450 inhibitor on gophers has the potential to increase the toxic effects of a toxin several fold, which would be a desirable control method for gophers.

Clotrimazole has been recognized as a potent ligand of the heme iron atom of cytochrome P450 enzymes. It has also been identified as a potent inhibitor for cytochrome P450 biotransformation enzyme in the possum (Olkowski et al., 1998) and rat (Turan, 2001). The present study evaluated the effect of clotrimazole on selected biotransformation phase I enzyme activities with respect to different gopher groups. IC_{50} values rather than K_i (the dissociation constant for inhibitor binding) values were

chosen to establish the inhibitory potency. The reason for this is that K_i values of an inhibitor can be overestimated when high microsomal protein concentration are used as a result of the depletion of the inhibitor by nonspecific binding to microsomal proteins (Kremers, 2002).

In our study, clotrimazole was identified as a potent inhibitor for the enzymes responsible for the metabolism of N-methylaniline, with a higher potency to exposed gophers than to naïve gophers. In this context, it is noteworthy that the co-administration of a potent inhibitor (clotrimazole) would lead to a considerable enhancement of bioavailability of N-methylaniline and, thus, increased toxicity. Since N-methylaniline is a toxin with high inherent toxicity, such a combined effect would eventually result in a significant increase in the toxicity of N-methylaniline in gophers, especially in those gophers previously exposed to toxins.

In addition to N-methylaniline, other compounds having various biotransformation pathways have also been used as probes to investigate the inhibitory potency of clotrimazole in gophers. Interestingly, N-methylaniline and N,N-dimethylaniline have been found to go through the same biotransformation pathway (N-demethylation); however, the inhibitory pattern of N,N-dimethylaniline metabolism is totally different to N-methylaniline metabolism inhibition. For example, naïve gophers were found to have higher IC_{50} values than exposed gophers regardless of gender for N-methylaniline metabolism inhibition, whereas for N,N-dimethylaniline metabolism inhibition exposed gophers tended to have higher IC_{50} values.

Moreover, the IC_{50} values were 2.7 times higher for N,N-dimethylaniline than for N-methylaniline. This suggests that in order to get the same inhibition effect for the metabolism of these compounds, a 2.7 times higher concentration of clotrimazole was needed to inhibit N,N-dimethylaniline biotransformation in comparison to N-methylamine biotransformation. In other words, when the same amount of inhibitor is added the inhibition effect on dimethylaniline is not as high as N-methylaniline.

Considering the fact that the K_m value of N-methylaniline is 2.6 times higher than N,N-dimethylaniline (see chapter 4), this indicates that enzymes responsible for

N,N-dimethylaniline metabolism have 2.6 times higher affinity to N-methylaniline. Thus, when the same amount of inhibitor and substrates on a molar basis is added, the biotransformation of N,N-dimethylaniline may be stronger than N-methylaniline. Furthermore, if the biotransformation result is detoxification (as in most cases) the potential toxicity of N-methylaniline will then be higher than that of N,N-dimethylaniline in the target animals.

Sex dependent differences in response to cytochrome P450 inhibition have also been observed for O-demethylation and aromatic hydroxylation using 7-methoxycoumarin and aniline as probes, respectively. It is important to note the potency of clotrimazole to female gophers is significantly higher than male gophers ($P<0.02$) for 7-methyoxycoumarin metabolism. However, the potency of clotrimazole in male gophers is significant higher than female gophers ($P<0.03$) for aniline metabolism.

Clotrimazole has also been identified as a potent inhibitor of cytochrome P450 enzymes in species other than gophers. Olkowski et al., (1998) showed that clotrimazole caused a marked inhibition of cytochrome P450 biotransformation activities in rats with IC_{50} values in the μM range for aromatic hydroxylation, N-demethylation and O-demethylation reaction.

In the present study, the inhibitory effect of clotrimazole on all studied cytochrome P450 catalyzed reaction in gophers was very potent with the IC_{50} values lower than 1 μM, except for aniline inhibition. For example, N,N-dimethylaniline N-demethylation, the IC_{50} of clotrimazole in chicken microsomal cytochrome P450 was reported to be 16.56μM, and in sheep is reported as 18.16μM (Olkowski et al., 1998). These values are considerably higher than in gophers that have an average microsomal cytochrome P450 of 0.57μM.

It is evident the inhibitory potency of clotrimazole in N,N-dimethylaniline N-demethylation reaction is higher in gophers in comparison to other animals. This finding is important for the control of gophers when considering non-target animal toxicity. The reason for this is the same amount of clotrimazole can strongly inhibit the detoxification of N,N-dimethylaniline through N-demethylation in gophers (target animals), but will

not significantly affect the detoxification of N,N-dimethylaniline through N-demethylation in other animals like sheep, rabbits and chickens. As such, metabolic characteristics of inhibitor clotrimazole and toxin N,N-dimethylaniline provide evidence that it is possible to design a toxicological strategy that will be more selective for gopher population control, and will not cause high toxicity to non-target animals. The same situation also occurs for 7-methoxycoumarin. For this compound, the inhibitory potency of clotrimazole was much lower in gophers (0.61μM) than in other animals such as chickens (15.66μM), rabbit (23.60μM) and sheep (18.01μM) which have been investigated in other studies (Olkowski et al., 1998). As for aniline, the differences between these species are less pronounced.

In addition to content and activity of cytochrome P450 enzymes (details see previous two chapters), we observed a considerable amount of interindividual variability in response to enzyme inhibition in our study. One potential reason for this is the variability is the basal constitutive expression levels of the cytochrome P450 system (for details see Chapters 3 and 4). However, other factors may contribute to the individual variability in response to enzyme inhibition in gophers.

In summary, it has been found that clotrimazole is a potent inhibitor for cytochrome P450 enzymes in gophers. As such inhibition of detoxification pathways may provide a logical approach to improve methods of gopher control in the future.

6.0 STUDY ON THE CYTOTOXICITY OF SELECTED COMPOUNDS USING FRESHLY ISOLATED GOPHER HEPATOCYTES

6.1 Introduction

Liver plays a key role in the metabolism of toxins. In addition to the basic information on detoxification reactions that can be obtained from the isolated cytochrome P450 enzyme system, the use of intact hepatocytes for *in vitro* studies offers an enhanced model, which can provide information whether the lower metabolic activity translates into a higher or lower potential for hepatotoxicity. A number of different *in vitro* liver models can be used in hepatic toxicity studies. The high sensitivity of these *in vitro* cytotoxicity tests may be due to absence of the protective mechanisms that assist cells within the body (Wallin and Arscott, 1998). For our purpose, freshly isolated hepatocytes represent a unique experimental approach for screening various compounds with regard to their cytotoxicity and it is the most frequently used *in vitro* model for the xenobiotic toxicity study (Wallin and Arscott, 1998). The use of hepatocytes in *in vitro* tests will increase the probability of detecting and characterizing the toxicity of our given compounds.

Cytotoxicity can be divided into two different groups: necrosis and apoptosis. Necrosis leads to cell lysis, followed by loss of cell constituents into its surroundings. In contrast, apoptosis is characterized by DNA condensation, nuclear fragmentation, and plasma membrane blebbing and cell shrinkage. Apoptosis characteristically occurs in isolated single cells. The duration of apoptosis is estimated to be from 12 to 24 hours, but in cell suspension visible morphologic changes are accomplished in less than two hours (Saraste, 1999).

A wide variety of toxic chemicals cause blebbing of the plasma membrane in isolated hepatocytes (Jewell et. al., 1982). These alterations in cell surface structure

occur well before cell death. Consequently, cell membrane blebbing is an early sign of cell injury and the most common symptom of acute cellular disease (Lemasters, 1983). Membrane blebs develop where a patch of the cell membrane has become disconnected from its lining of cytoskeleton (Dai and Sheetz, 1999). Losing cytoskeletal support, the cell membrane bulges outward due to intracellular pressure (Majno and Joris, 2004). The formation of blebs appears to be directly related to changes in the concentration of extra mitochondrial calcium ions. These changes probably reduce the ability of the hepatocyte cytoskeleton to maintain normal surface morphology.

Plasma membrane blebs may eventually lead to the rupture of the bleb or bleb shedding. In bleb rupture, the bleb will undergo lysis, cell necrosis and directly release cytoplasmic constituents into the circulation or surrounding tissue. With the reversible injury, the cytoplasmic constituents can be released by shedding of cytoplasmic fragments through budding off of blebs and the plasma membrane will be reseated. Despite the importance attributed to bleb formation during necrotic cell injury, the exact mechanism causing bleb formation is still not clear.

The trypan blue exclusion test is widely used to estimate the percentage of viable cells in isolated hepatocyte suspensions (Guillouzo, 1998). Trypan Blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all cells that exclude the dye are viable; cells dyed blue are nonviable. A hematocytometer is used to analyze the mixture under a microscope and the fractional viability is easily calculated by dividing the number of clear cells by the total number of cells.

The current study was aimed at evaluation of the *in vitro* cytotoxic potential of selected compounds using freshly isolated gopher hepatocytes. The compounds tested included coumarin, 7-methoxycoumarin, aniline, N-methylaniline and N,N-dimethylaniline. Furthermore, the cytotoxicity potential was evaluated in the presence and absence of a general cytochrome P450 inhibitor clotrimazole to confirm the cytotoxicity as a result of the parent compound and possible protective function of

cytochrome P450 enzyme activity.

6.2 Materials and Methods

Hepatic cell culture preparation: Gophers were trapped from various locations on University of Saskatchewan campus. Liver tissues were harvested immediately after the animals were euthanized according to the protocol described in detail in Chapter 3, and processed at 4^{o}C. The livers were removed and rinsed thoroughly, and then immediately perfused with ice cold 0.1M Phosphate buffer (pH 7.4). A piece of a lobe was sectioned, and further used for hepatocytes isolation. Iscove's Modified Dulbecco's medium (IMD) containing 0.1 g/L collagenase (Collagenase IV, Sigma-Aldrich Co. Louis, U.S.A.) was used for tissue digestion. The entire procedure was carried out under sterile conditions. The medium was delivered to the tissues via peristaltic pump. After the peristaltic pump (Tris^{TM}, ISCO. Inc., Nebraska, U.S.A.) was started, a sterilized cannula was introduced into the main vein of the separated lobe. The liver tissue was then perfused for 30 min with collagenase IMD media at a flow rate of 60 ml/min at 37 ^{o}C. After the digestion, the liver tissue was removed from the apparatus and placed in ice cold 50 ml IMD media. The liver was then minced in the beaker with a pair of sterile scissors to release the hepatocytes into the media. Next, the cell suspension was filtered (cell strainer, sterile, 70 um Mesh, Falcon, BD#352350, U.S.A.) to separate the hepatocytes. The media was then centrifuged in sterile 15ml centrifuge tubes (500 rpm for 3 minutes at 4 ^{o}C). The supernatant was discarded and the pellet of hepatocytes was then rewashed with IMD media 3 times followed by re-suspension in IMD media.

Cell viability assay: 4% (w/v) Trypan Blue exclusion test was used to determine the viability of isolated hepatocytes. 5µl of the 4% Trypan Blue was mixed with 100µl of the cell suspension, and the mixture then incubated at the room temperature for at least 5 minutes. Cells were then loaded onto a hematocytometer (a counting chamber covered with a cover slide) and counted under a microscope. Cells that did not absorb stain and had membranes intact with shine silver color were regarded as viable cells. Cells with multiple surface protrusions were considered as damaged cells or blebbing cells. Cells with a clearly stained nucleus were counted as dead cells.

Incubation: The cells were diluted to the same cell concentration (about 1 x 10^5 cell/ml) and were cultured on 96 wells micro-plates. 95µl of the cell culture and 5µl of the substrates (Table 6.1) were then mixed thoroughly and the micro-plates were covered and incubated at 37°C. Controls were prepared with 95µl cell culture and 5µl methanol and treated identically as the samples were. On a separate plate, substrates (around 0.625 x K_m value for each substrate) with or without the inhibitor (1µM) were added to the cell culture. The plate was then covered and incubated under 37°C. Incubations were terminated after 2 hours, and 5µl of 4% Trypan Blue was added to the 100µl reaction mixture. Following this, the cell culture was examined under the microscope. The cells were counted on randomly selected microscopy fields. For this studies purpose, the numbers of cells showing morphological change as well as dead cells were then enumerated.

Table 6.1. Concentrations of various compounds: 5, 10, and 20 times of the K_m value of each compounds. These compounds included coumarin, aniline, N-methylaniline, N,N-dimethylaniline, and 7-methoxycoumarin, which are substrates of cytochrome P450 enzymes. K_m values were determined as described in chapter 4.

Substrate Concentration	Coumarin	Aniline	NMA	DMA	7- MC
20 x K_m	0.34mM	0.81mM	6.1mM	2.48mM	0.12mM
10 x K_m	0.17mM	0.41mM	3.05mM	1.24mM	0.06mM
5 x K_m	0.09mM	0.20mM	1.525mM	0.62mM	0.03mM

6.3 Method Validation

In this study, 3 gophers were used to isolate hepatocytes for hepatic toxicity evaluation. The preliminary experiments showed that the optimal cell density and culture substratum were found to be 100 µl of 1 x 10^5 cell/ml per well of a 96-well plate. Viabilities of hepatocytes (Figure 6.1) of all three gophers on the plates were as follow: 90.3% for gopher #1; 85.2% for gopher #2; 84.4% for gopher #3.

Hepatocytes were incubated with the highest concentration of each substrate, and the incubation was ended after 2 hours and 4 hours separately. The cell mortality for these two time endpoints was similar. Therefore, 2 hours was chosen as the time factor for incubation for all experiments.

The time for dye reaction with cell suspension was evaluated and 5 min was found to be the minimum time for the cell to settle down and for hepatocytes staining. Optimization of the dye reaction duration is critical for cell counting since the cells will settle down as one layer and the dead cells will be clearly distinguished from the viable cells. Cell viability was checked through cross-reference evaluation. In some cases, 3 operators counted the cell variability separately with consistence reached through calculating the mean of the operators cell count.

6.4 Results

The cytotoxic effect of selected substrates in freshly isolated hepatocytes from gophers was evidenced through morphological examination and from the results of the Trypan Blue test. Both cell blebbing (Figure 6.2) and cell death (Figure 6.3) were observed in these toxicological studies. Cell mortality calculations considered those cells with a stained nucleus to be dead (Figure 6.4).

At high concentration, all these substrates exhibited a significant cytotoxic effect on the hepatocytes from all three gophers. In addition, these effects were concentration-dependent (Table 6.1). Some cells also developed small blebs on the cell membrane (Figure 6.5). As mentioned previously, Table 6.2 indicated a rough dose response trend in cell mortality. When toxin concentration was increased a corresponding increase in

hepatocyte mortality was observed in all the selected substrates (Figure 6.6). However, methanol solvent accounted for part of the cell death since the viability of hepatocytes incubated with methanol (68%) was lower than the viability of the freshly isolated hepatocytes (>80%).

Table 6.3 provides a description of the effect of inhibitor clotrimazole (CTZ), a cytochrome P450 inhibitor. The cytotoxic effect for all the selected substrates demonstrated an increased in the presence of CTZ. Furthermore, CTZ itself was also toxic to the hepatocytes. Thus, a synergistic effect on hepatotoxicity was observed when comparing hepatotoxicity of substrates and inhibitor separately with substrates and inhibitor together.

Two types of hepatic toxicity were observed in this study. Hepatic cell death with very few cell blebbing occurred when aniline and its derivatives were applied to the hepatocytes. However, there were considerably higher percentages of damaged cells with dominant cellular membrane blebs in the incubation mixture of hepatocytes and coumarin or coumarin derivatives (Table 6.4). Interestingly, the combination of CTZ and coumarin or 7-methoxycoumarin had a lower percentage of cell blebbing than the CTZ itself. However, the combination had higher cell death than the separated ones (Table 6.4).

Figure 6.1. Viable hepatocytes freshly isolated from gopher's liver. The viable cells excluded the trypan blue, which is evident from the characteristic features such intact cellular membranes with shiny silvery color. Original magnification 400x, digital magnification 3x).

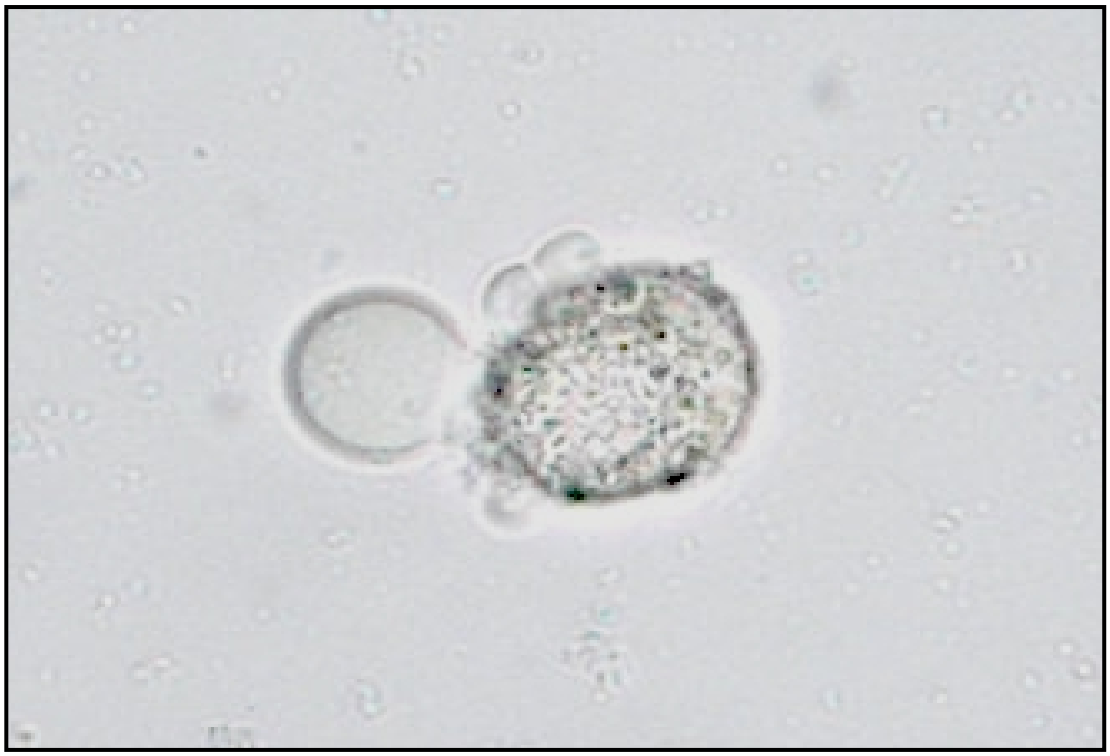

Figure 6.2. Hepatocyte showing morphological changes. Cells with multiple cellular membrane protrusions (plasma membrane blebbing) were considered as injured in association with cytotoxicity of administered compounds. Original magnification 400x, digital magnification 4x).

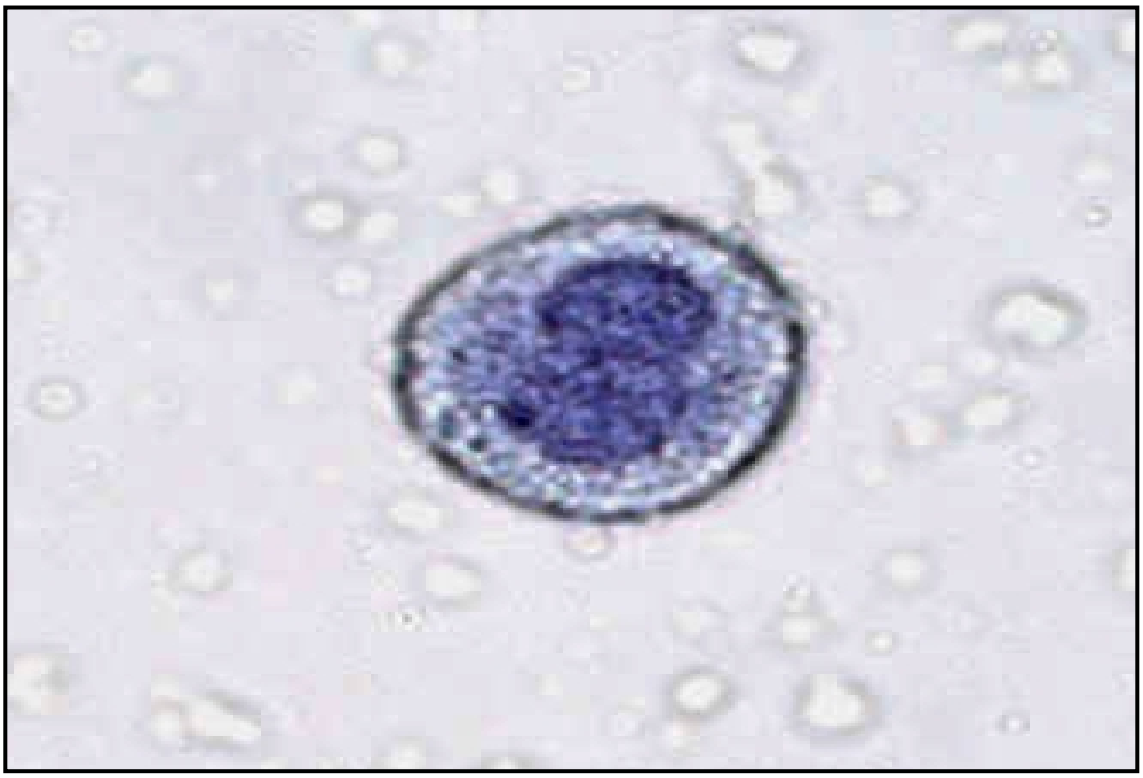

Figure 6.3. An example of not viable hepatocyte. Noteworthy is clearly stained nucleus indicative that the cell was unable to exclude trypan blue dye. Original magnification 400x, digital magnification 6x).

Figure 6.4. Different stages of gopher hepatocytes death. The cell on the left shows mildly blue stained nucleus, which indicates that this cell was in the process of dying, whereas the features seen in the cell on the right (strongly stained nucleus and cytoplasm), indicate that the cell was dead for some time. Original magnification 400x, digital magnification 6x.

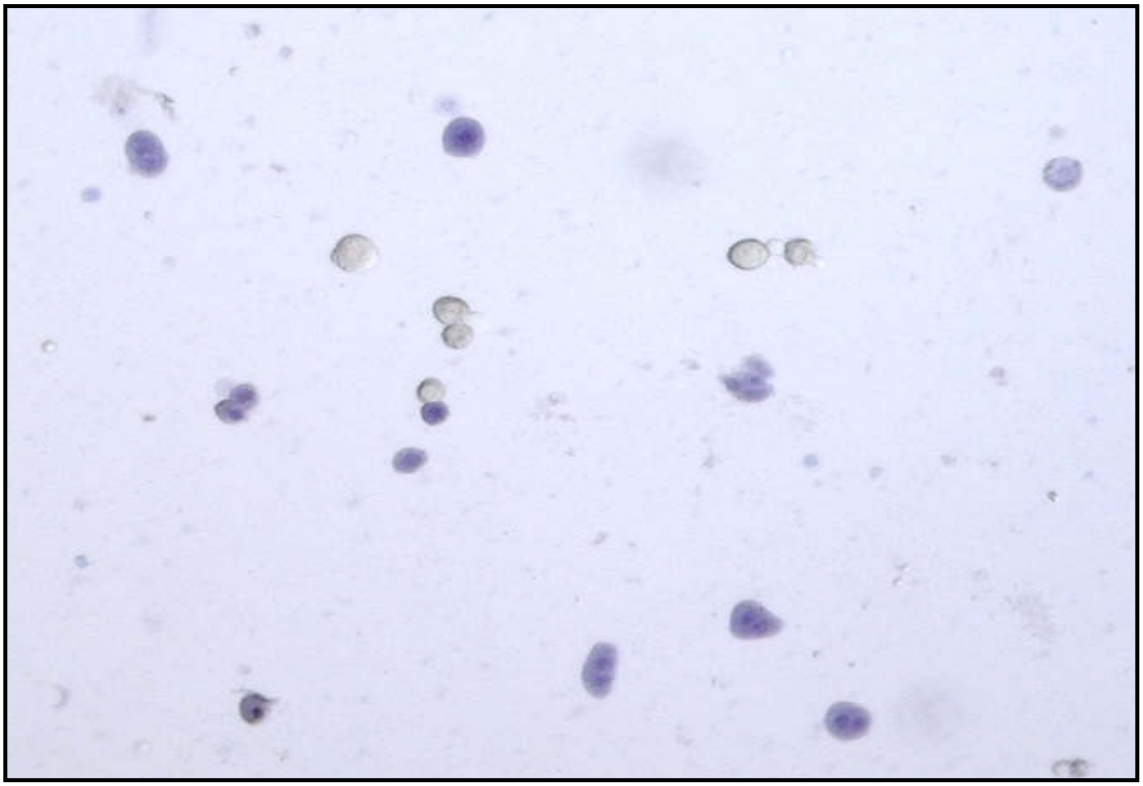

Figure 6.5. Hepatoxicity associated with aniline. Trypan blue exclusion test was used to distinguish viable and dead cells. It is noteworthy that the majority of the stained hepatocytes died without blebbing, which indicated that cell death resulted from acute toxicity. Original magnification 100x, digital magnification 3x).

Table 6.2. Mortality of hepatocytes (percentage of dead cells) incubated with different concentrations of various substrates (Mean ± SE) (n=3).

Toxin concentrations (μM)	Percentage of cell death (%)				
	Aniline	NMA	DMA	Coumarin	7-MC
20 x K_m	56 ± 0.58^{a}	46 ± 4.26^{a}	52 ± 4.48^{a}	40 ± 4.51^{a}	42 ± 4.16^{a}
10 x K_m	44 ± 1.20^{b}	40 ± 3.61^{ab}	41 ± 4.26^{b}	37 ± 1.15^{ac}	33 ± 1.45^{b}
5 x K_m	36 ± 0.88^{c}	37 ± 3.51^{ab}	34 ± 2.31^{b}	28 ± 0.58^{b}	33 ± 0.88^{b}
Control	32 ± 0.58^{d}	32 ± 0.58^{b}	32 ± 0.58^{b}	32 ± 0.58^{bc}	32 ± 0.58^{b}

The values are means of three animals (SE=standard error). Means were compared using Fisher's LSD, $\alpha = 0.05$. Means with the same letters are not significantly different.

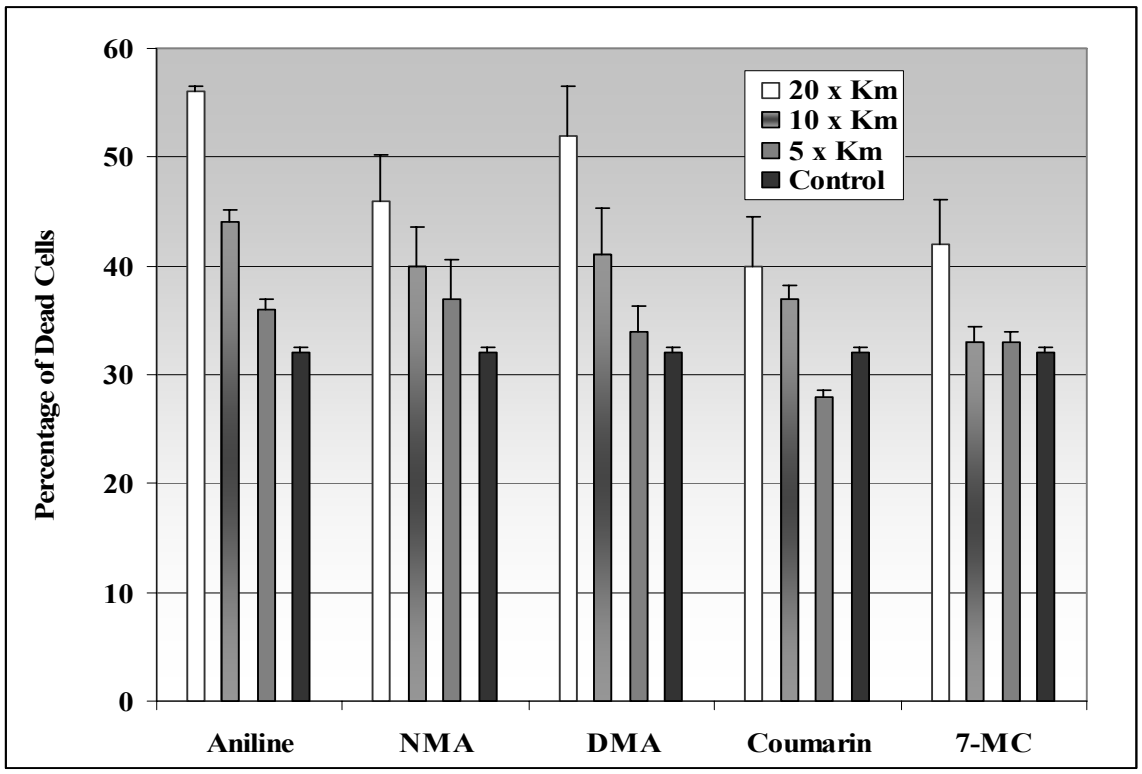

Figure 6.6. Dose-response trends for the effect of all the selected substrates on hepatocyte mortality. Isolated hepatocytes were exposed to these compounds for 2h. Values are mean (±SE) of 3 individual gophers.

Table 6.3. Comparison of the hepatocyte mortality of different substrates with and without inhibitor and the effect of the inhibitor itself (n=1)

	Percentage of cell death (%)				
Substrate	Aniline	NMA	DMA	Coumarin	7-MC
0.625 x K_m	40	40	39	37	40
0.625 x K_m + CTZ	47	57	49	58	53
CTZ only	39	39	39	39	39
Control	32	32	32	32	32

Table 6.4. The percentage of the damaged cell when incubated with different concentration of coumarin and 7-methoxycoumarin and the effect of inhibitor (Mean ± SE).

	Percentage of cell damaged	
Substrate	Coumarin	7-methoxycoumarin
20 x K_m	23 ± 2.89	24 ± 2.03
10 x K_m	13 ± 1.20	11 ± 1.53
5 x K_m	10 ± 1.45	6 ± 1.20
0.625 x K_m	0	6
0.625 x K_m + CTZ	10	9
CTZ only	18	18
Control	0	0

The values for 20 x K_m, 10 x K_m and 5 K_m are means of three replications (SE= standard error). The rest of the values are from one individual.

6.5 Discussion

The liver is the principal organ involved in the biotransformation of exogenous substances. However, more than a thousand xenobiotics are potentially hepatotoxic. Freshly isolated hepatocytes, which retain most liver-specific functions, represent a valid and simplified model for predicting the hepatotoxicity of xenobiotics (Wallin and Arscott, 1998). They possess the full spectrum of xenobiotic metabolism enzymes, cofactor and cell membrane receptors. In addition, the compound tested can be rapidly dispersed throughout incubation and accessible to all hepatocytes in the incubation phase (Griffin and Houston, 2004). However, due to their loss of viability within 4 hours and a decrease in cytochrome P450-dependent metabolism upon culture (Griffin and Houston, 2004), hepatocyte toxicity experiments represent a short period of a toxicity investigation.

In this study hepatotoxicity was observed when selected substrates were incubated with freshly isolated hepatocytes. The cytotoxicity of all selected toxins was found to be concentration-dependent. When the concentration of the toxin incubated with freshly isolated gopher hepatocytes increased, the toxicity as assessed by cell responses, also increased. Thus, there is a correlation between compound concentration and cell response with regard to the cytotoxicity. However, because of the limited number of data points, the present study does not allow for complete evaluation a dose-response relationship.

Studies suggest coumarin hepatotoxicity is mediated by the production of a reactive epoxide catalyzed by the cytochrome P450 system (Fentem and Fry, 1993). Given this, it can be anticipated that the inhibition of cytochrome P450 system may reduce hepatotoxicity. However, in this study, the presence of the inhibitor increased the cytotoxicity of coumarin as well as other substrates. Therefore, it is possible that other mechanisms of hepatoxicity associated with coumarin occurred in our model. Considering the fact that incubation with the inhibitor also only caused certain extent of cell death, these increased cytotoxicities may be the results of synergistic or additive effect of the inhibitor and the selected substrates of cytochrome P450 enzymes.

When comparing the cytotoxicity of aniline with N-methylaniline and N,N-dimethylaniline; aniline was found to be more toxic. At the highest concentration (0.81 mM), aniline caused 56% hepatotoxicity, and at the lowest concentration (0.20 mM), aniline still caused 36% of cell death. The highest concentrations used in this experiment for methylaniline and dimethylaniline were 6.1mM and 2.48 mM respectively. These concentrations were much higher than that used for aniline. However, cell deaths caused by these chemicals and concentrations were only 46% and 52%, respectively (Table 6.2). This is consistent with the fact that detoxification rate of aniline by cytochrome P450 enzymes is much lower than N-methylaniline and N,N-dimethylaniline (for details see Chapter 4).

There were distinctly different types of hepatotoxic effects observed in the present study. For instance, coumarin and 7-methoxycoumarin mainly induced plasma membrane blebbing, a protrusion of cell, shaped like blisters or balloons attached to the cell by a narrow neck (Figure 6.3). Whereas, aniline and aniline derivatives mainly induced cell death with few cell blebbing. Blebbing occurs very quickly after cell damage, which allows it to be a very sensitive indicator of cellular injury. In our study, cell blebbing occurred after two hours of incubation with various toxins. However, it should be noticed that blebbing is not necessarily a fatal event for the cell since this kind of change may be reversible (Schwartz et al., 1984). Bleb formation during hepatocyte injury is generally described in three stages (Rosser and Gores, 1995). During the early stage of cell damage, numerous small blebs appear on the plasma membrane (Stage I). As the damage continues, the small blebs enlarge by coalescence and form a few large terminal blebs (Stage II). Up to stage II, the plasma membrane still has normal barrier function (Gores et al., 1990) and the bleb formation is reversible and resorption can occur if the insult is reversed (i.e. remove the toxic compounds) (Rosser and Gores, 1995). The last stage (Stage III) includes loss of the plasma membrane permeability barrier, which is synonymous with cell death (Gores et al., 1990). Thus, the toxicity of coumarin or 7-methoxycoumarin can be reversed by removing the source of toxins, whereas, the toxicity caused by aniline, N-methylaniline and N,N-dimethylaniline will be permanent. Considering bait shyness phenomenon in rodent control, this finding has practical significance. For instance, toxins such as aniline, N-methylaniline and N,N-

dimethylaniline will likely cause substantial damage to the hepatocytes of gophers, even following a single exposure to the toxin. However, poison baits containing coumarin or 7-methoxycoumarin, and likely the entire class of coumarin congeners commonly used in pest control may not be efficient since the toxicity may be reversed once the animal (e.g. gopher) can associate their illness with the bait which will lead to bait shyness.

In conclusion, the results from the isolated hepatocytes indicate that some compounds can cause acute, and likely the terminal effect of cell death when applied in sufficient quantities, whereas other compounds may result in chronic, and possible reversible effects.

Overall, this short term *in vitro* cytotoxicity test could be used to help to screen for wide range of toxins, and to select the *in vivo* starting dose for an acute lethality assay. The use of long-term hepatocyte cultures might be the next procedure since it permits chronic effects to be monitored. Based on the observation from our studies, further investigations using the methods employed here are warranted.

7.0 GENERAL DISCUSSION AND CONCLUSION

The problems caused by gophers are primarily faced by those involved with forage or livestock production and these problems have been evident since people began settling on the prairies. As such an extensive amount of resources have been committed to control the gopher population. Physical methods such as trapping and shooting are time consuming and laborious. Chemical methods, which include various poison containing baits, are considered to be more effective methods to control gopher populations.

Chemical toxicants such as strychnine and zinc phosphide and anticoagulants can result in an immediate high mortality (Michener and Koeppl, 1985; Proulx, 1998), and are conventional chemical methods of gopher control (Lund, 1985). However, less than 31% of farmers are satisfied with their gopher control efforts according to a recent report commissioned recently by the government of Saskatchewan (2001 Saskatchewan "Gopher" Survey).

During our own investigations, we counted gophers on a field where no toxins were used (site A) and on the field where toxicological controls were in place (site B). Interestingly, both sites were severely infested with gophers and we observed no apparent difference in gopher density between the two fields. Both sites contained approximately 75 to 100 gophers in approximately 1 hectare. Our observations are consistent with the conclusions of 2001 Survey suggesting that the current chemical methods of gopher control may not be effective. A reason for this is it could be possible that gophers previously exposed to toxic baits in many areas of the province may have developed resistance to toxins used, which has caused a significant impediment in the attempts to control gopher populations. Consequently, more effective gopher control methods require development, and chemical methods seem the most convenient for the producer.

Currently no scientific data is available to explain this apparent lack of efficiency of the poison control methods in gophers. However, acquired resistance to toxins is a strong possibility, since studies in alternative species indicate resistance in pests is generally an inadvertent outcome of pesticide application, and has caused the loss of many formerly efficacious pesticides (Brown, 1991). Strychnine, which in the past had been an effective compound for gopher control, has been losing its efficiency likely due to such acquired resistance (Saskatchewan gopher survey: final report, 2001).

Animals may develop resistance to toxins by three basic mechanisms: 1) Behavioral and physiological mechanisms (Devine and Cook, 1998), 2) Protein products involved in resistance (Agosin, 1982), and 3) Genetic mechanisms conferring resistance (Feyereisen et al., 1989). Despite the lack of available scientific data regarding behavioral avoidance of the pesticide in gophers, during trapping we observed that gophers approached the peanut butter bait provided in the trap with great caution. The observed behavior (i.e. gophers approached the trap very carefully, looked around, tapped the trap door, went away and came back after sometime) was very consistent between different individuals. Such behavior indicates a highly developed self-preservation instinct.

Regarding the second mechanism our studies indicated that the content and activity of individual components of the cytochrome P450 system including cytochrome P450, cytochrome b5 and NADPH-cytochrome P450 reductase in liver microsomal preparations were higher in toxin exposed gophers and varied greatly between gophers of different gender. Consequently, gophers exposed to toxins used to control their population had a generally higher detoxification capacity than did naïve gophers. This suggests that gophers previously exposed to toxins may readily develop resistance to chemicals by enhancing the biotransformation potential.

Finally, the resistance to toxins can be transferred genetically, where innately tolerant individuals pass on to their offspring the genes responsible for resistance (Brown, 1991). These changed genes conferring enhanced resistance to a pesticide (e.g. cytochrome P450s) become common genes in the population, since they confer a

survival advantage. With continued exposure, a persistent genetic change may occur at the population level (Brown, 1991). It should be noted; however, that in our study, the sampling process was in the middle of July when most adults went into hibernation (Michener, 1995). Therefore, it is reasonable to assume that gophers used in the present study were juveniles. Thus, the enhanced biotransformation potential could have been inherited from the parents previously exposed to the poison.

As mentioned previously, strychnine has been used to control the gopher population in the field where we trapped toxin exposed gophers. Strychnine is metabolized primarily through hydroxylation and N-oxidation by the hepatic microsomal enzyme system (e.g. CYP2B in rat) (Fujisaki et al., 1994; Mishima et al., 1998; Health Council of the Netherlands, 2004). As such, content of cytochrome P450 system in gophers will determine the oxidation extent of strychnine and, consequently, the toxicity of the compound to the animal. Worth mentioning is the fact that strychnine has been identified to be a potent inducer of hepatic P450 metabolizing enzymes in rats (Fujisaki et al., 1994). Similarly, it is possible that the increased content of cytochrome P450 systems found in exposed gophers may have been associated with strychnine induction, which might finally result in extensive metabolism and a corresponding reduction in strychnine toxicity. If this hypothesis is correct this would be an adaptive response that protects gophers from strychnine toxicity.

Interestingly, strychnine administration in the drinking water of rats significantly increased hepatic microsomal activities of benzphetamine N-demethylation (Fujisaki et al., 1994). In our study we found that gophers previously exposed to strychnine showed lower hepatic microsomal activities of N-methylaniline and N,N-dimethylaniline N-demethylation than naïve gophers. This inconsistency in the same biotransformation pathway may be caused by interspecies differences in the expression of biotransformation enzymes, a typical phenomenon in cytochrome P450 associated metabolism (DeBethizy and Hayes, 1989).

From an evolutionary standpoint, xenobiotic dependent induction of cytochrome P450 enzymes in gophers is very important. Cytochrome P450 induction usually

enhances detoxification. Hence, under most conditions, induction is a protective mechanism (Klassen, 2001). Furthermore, induction is likely to be advantageous in the survival of a species as it allows enhanced detoxification following exposure to xenobiotics by accelerating the inactivation and clearance of potentially lethal xenobiotic compounds. This type of induction may also be inherited by offspring which could magnify its importance for the survival of the species. For example, strychnine can cause profound imprinted overinduction of cytochrome P450 isoenzymes CYP2B1 and CYP2B2 in rat (Agrawal and Shapiro, 1996), which may allow this species built up resistance after several generations. However, whether strychnine induction can be passing on to the juvenile gophers is not clear since there is no scientific data available with respect to the CYP450 isoforms in gophers.

Other than the toxic compounds used to control the population of gophers, dietary components and environmental pollutants may also cause induction of cytochrome P450 enzymes. In our study, the gopher diets were not extremely variable among individuals, and the compositions of diets was similar for exposed and naïve gophers. Consequently, exposure to chemical control agents likely explains the observed enhancement in cytochrome P450 enzyme system.

However, the currently used gopher control method may still have some effects on the biotransformation enzymes in gophers. The *in vitro* cytochrome P450 mediated activities for five substrates [coumarin and aniline aromatic hydroxylation, 7-methoxycouamrin O-demethylation, and N-methylaniline, N,N-dimethylaniline N-demethylation] were higher in naïve gophers than gophers exposed to toxins previously. But naïve gophers have similar whole body activity as exposed gophers. In some cases, exposed gophers even have a slightly higher whole body activity than naïve gophers. This indicates that gophers may compensate the loss of the enzyme activity by increasing the content of the detoxification enzymes, which may eventually lead to the resistance to the control method.

In conclusion, a very plausible explanation why the current methods of gophers control are ineffective is the increased capacity of biotransformation acquired upon

exposure to toxin. This can be supported by our study. Gophers increased their detoxification capacity by the enhancement of components of the main detoxification system despite the activity of the enzyme decreasing when gophers were exposed to the toxin and behavioral avoidance to pesticide.

8.0 PROPOSED STRATEGIES TO IMPROVE GOPHER CONTROL IN SASKATCHEWAN

There are three responses to pesticide resistance: 1) increasing the amount of pesticide, 2) increasing the frequency of application, or 3) rotation of pesticides used (NRC, 1986). The first two solutions are self-defeating since they tend to exacerbate the development of resistance. The third solution may ultimately cause its own resistance problems, if there is no basic difference in the mode of action and treatment strategy (Roush, 1981). Therefore, a better alternative for toxicological control of gophers avoiding resistance is needed.

Resistance can be associated with enhanced activity of the cytochrome P450 detoxification system (Brown, 1991). This is apparent from the present findings that gophers previously exposed to toxins showed enhanced detoxification capacity by increasing the content of hepatic cytochrome P450 systems when they were exposed to toxin though their specific activities were decreased. Based upon the findings of this study, several possible strategies for gopher control will be proposed.

8.1 Possible Strategies to Control Gopher Populations

8.1.1 Exploit Gender Dependent Differences

Many animals exhibit sex dependent differences in hepatic xenobiotic metabolism (Pampori and Shapiro, 1999). Gophers are no exception. This study showed that male gophers have higher aromatic hydroxylation, O-demethylation or N-demethylation activity than corresponding female gophers. We can exploit these gender-associated differences for practical gopher control. Since in most cases cytochrome P450 enzyme mediated reactions lead to detoxification of xenobiotics, a compound with relatively high inherent toxicity that undergoes aromatic hydroxylation or demethylation

as the principal detoxification pathways may result in enhanced toxicity in female gophers. This may offer an attractive approach to gopher control, since females represent the majority of the adult gopher population, with male to female ratio 1:3 (Michener, 1979; 1995). Selective elimination of females may effectively decrease gopher population size since the average female gives birth to 6 to 9 juvenile gophers per year (Sheppard, 1972; Michener, 1995).

However, it is also well known that some cytochrome P450 enzymes can convert low toxic parent compounds into more toxic metabolites. In this situation, a compound with relatively low inherent toxicity can be metabolized to metabolites that are more toxic than the parent compound. Given the males enhanced xenobiotic metabolism capacity such compounds will lead to greater toxicity in male gophers. Considering the fact that natural mortality among the male Richardson's ground squirrels is quite high and the adult sex ratio is about three or four females to one male regardless of the geographic location, year, or population density (Michener, 1979; 1995), further reductions in male populations will reduce the reproductive capacity of the population as a whole.

Cytochrome P450 enzymes may also catalyze activation and detoxification reactions for a given chemical at the same time. In this case, the balance between metabolic activation and deactivation will determine the toxic effect of a xenobiotic chemical (Casarett and Doull, 1996) and the results of such a balance will be very complex.

8.1.2 Sub-population analysis

This study identified a considerable degree of individual variability in cytochrome P450 enzyme activity in the gopher population. Variability of biotransformation reactions is very common, the rule rather than the exception. In the present study we identified a sub-group of gophers, which showed lower detoxification capacity than the rest of the population. This subgroup represented approximately 25-42% of the population. For this subgroup, if a toxin is used that has inherent toxicity and is detoxicated through the same metabolic pathway as aniline, coumarin or 7-

methoxycoumarin, then 25%-42% of the gopher population would be more susceptible because of lower inherent detoxification potential. This apparent metabolic feature could be exploited as a potential biochemical weakness for the control of the gopher population. In addition to this, if a toxin can be activated through the same metabolic pathway as aniline, coumarin or 7-methoxycoumarin, 58%-75% of the population could be targeted; this is also a desirable method for gopher population control.

8.1.3 Kinetic Characteristics of Different Biotransformation Pathways

The present study investigated several different biotransformation pathways including N-demethylation, O-demethylation and aromatic hydroxylation. The results indicated that the K_m values for N-demethylation were higher than aromatic hydroxylation for both naïve and exposed gophers; while, K_m values for O-demethylation were the lowest. On the other hand, the V_{max} value based on cytochrome P450 content for demethylation reactions were higher than aromatic hydroxylation reactions. These kinetic features can also be exploited to provide a better method to control gophers.

For example, N-methylaniline has the highest K_m value, which indicates it can accumulate in the body at high concentration prior to reaching the highest metabolism speed. As such, N-methylaniline will become more distributed throughout the body eventually causing adverse effects and death, which is desirable in a rodenticide developed for gopher control. Conversely, O-demethylation enzymes have the lowest K_m values, which indicate when the toxin gets into the system, they will be metabolized even if there is just a little substrate. Whereas, if a compound goes through a similar biotransformation pathway as 7-methoxycoumarin and has a low K_m value but a low inherent toxicity, it will be biotransformed to toxic metabolites quickly, and the accumulation of such toxic metabolites will be desirable in controlling the gopher population.

Additionally, toxins with high inherent toxicity and relatively low metabolite toxicity undergo aromatic hydroxylation, low V_{max} will result in adverse effects and death. For toxins with low inherent toxicity but with highly toxic metabolites and it go

through N-demethylation, high V_{max} will result in high toxicity. Both of these toxins will then be desirable toxins to be used to control gopher population.

8.1.4 Combination of Pesticides

A combination of the possible toxic agents with a potent inhibitor might be a valuable rationale for the development of the new pesticide combination in the future. In this context, a concept of "pesticide cocktail" comprised of several pesticides, and including some specific inhibitors of biotransformation, is worthy of thorough examination.

As previously mentioned the liver is the main organ responsible for xenobiotic biotransformation. Specific inhibition of biotransformation enzymes in gopher liver will result in impaired metabolism (for details see chapter 2). Hence, the inhibitor will enhance the toxic potential of a pesticide associated with first pass biotransformation. Bioavailability and systemic exposure will increase, correspondingly, and target animals will manifest exaggerated responses to the toxic compounds used in the control of the gopher population.

Furthermore, a rodenticide can be developed based on the fact that gophers spend majority of their life underground in hibernation (Michener, 1998; Michener, 2002). In this case, a small amount of highly lipophilic compound with high inherent toxicity that undergoes metabolism in liver and is eliminated as water soluble metabolites with low toxicity, can be accumulated in body (e.g. fat tissue) during summer time and during hibernation fat will be mobilized and the compound will be released into the system. Under this circumstance, the accumulation may inevitably progress to the point of high toxicity, which would thus cause gophers to die during hibernation. This would therefore decrease secondary toxicity to the non-target animals since the inhibitor has higher potency to gophers than other target animals and a small amount of certain toxins with such an inhibitor will have less primary risk to non-target animals as well. However, in the situation of specific inhibition of certain metabolism pathway (i.e. N-demethylation, O-demethylation or aromatic hydroxylation), alternative pathways of metabolism can occur, which may affect the final outcome of the co-administrated

compounds since the normally minor pathway may become of major importance when the normal main pathway is relatively unavailable. Therefore, a general inhibitor for all cytochrome P450 enzymes will be suitable in both situations. This concept has also been identified in the cytotoxicity test using freshly isolated hepatocytes where the addition of inhibitor, clotrimazole, increased the cytotoxicity of the selected compounds.

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Appendix I: ISCOVE'S MODIFIED DULBECCO'S MEDIUM

Components	g/L
Calcium Chloride (anhydrous)	0.1653
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.33
Potassium Nitrate	0.000076
Sodium Chloride	4.505
Sodium Phosphate Monobasic (anhydrous)	0.109
Sodium Selenite	1.73E-05
L-Alanine	0.025
L-Arginine•HCl	0.084
L-Asparagine• H_2O	0.0284
L-Aspartic Acid	0.03
L-Cystine•2HCl	0.09124
L-Glutamic Acid	0.075
L-Glutamine	0.584
Glycine	0.03
L-Histidine•HCl• H_2O	0.042
L-Isoleucine	0.105
L-Leucine	0.105
L-Lysine•HCl	0.146
L-Methionine	0.03
L-Phenylalanine	0.066
L-Proline	0.04
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016

L-Tyrosine•2Na•2H_2O	0.10379
L-Valine	0.094
D-Biotin	0.000013
Choline Chloride	0.004
Folic Acid	0.004
myo-Inositol	0.0072
Niacinamide	0.004
D-Pantothenic Acid (hemicalcium)	0.004
Pyridoxal•HCl	0.004
Riboflavin	0.0004
Thiamine•HCl	0.004
Vitamin B_{12}	0.000013
D-Glucose	4.5
HEPES	5.958
Phenol Red•Na	0.016
Pyruvic Acid•Na	0.11